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Dissociating Siv Env and Cd4: Consequenes for Virus and Host

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Abstract
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I first describe the derivation of iMac239, a CD4-independent variant of SIVmac239. Like other CD4-independent variants, we found that a mutation in the V1/V2 loops of Env was required for CD4-independent entry, and that acquisition of CD4-independence resulted in an increase in neutralization sensitivity. While iMac239 was CD4-independent, its CD4-binding site was intact, thus we removed the Aspartic Acid residue at position 385 (analogous to D-368 in HIV-1) to ablate CD4 binding. We found that this novel variant, iMac239-ΔD385, exhibited replication kinetics similar to that of the parental iMac239 strain, and was insensitive to neutralization by soluble CD4. Both CD4-independent strains exhibited an expansion of cellular tropism in vitro with infection of CD4- CD8+ T cells in stimulated rhesus PBMCs.

Next, I present our evaluation of iMac239-ΔD385 pathogenesis and immunogenicity in four rhesus macaques. iMac239-ΔD385 replicated to a high acute viral peak, but was quickly controlled to undetectable levels by the host immune response. iMac239-ΔD385 infection elicited high and sustained neutralizing antibody titers and polyfunctional T cell responses. Strikingly, we observed an alteration in the distribution of infected cells in the lymph node and expansion in the types of cells infected. We tested iMac239-ΔD385 as a live attenuated vaccine against a pathogenic SIVsmE660, and while the number of animals in the study is too small to determine significance we observed a trend toward improved outcomes post challenge, potentially due to a synergistic interaction between iMac239-ΔD385 vaccination and Trim5α alleles.

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DISSOCIATING SIV ENV AND CD4: CONSEQUENCES FOR VIRUS AND HOST

Adrienne E. Swanstrom

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ABSTRACT

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James A. Hoxie, M.D.

CD4 tropism is conserved among all primate lentiviruses and likely contributes to viral pathogenesis by targeting cells that are critical for the adaptive anti-viral immune responses. Although CD4-independent variants of HIV and SIV have been described that can utilize coreceptors CCR5 or CXCR4 in the absence of CD4, these viruses typically retain their CD4 binding sites and can still interact with CD4. In this thesis, I present the characterization and evaluation, both in vitro and in vivo, of a novel CD4-independent variant of SIV lacking a CD4 binding site.

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CHAPTER 1

General Introduction

Overview

In 30 years of the HIV-1/AIDS pandemic great advancements have been made in the management of infection and disease, from a diagnosis of HIV-1 infection serving as a death sentence in the 1980s to a chronic but manageable disease in the 2000s. The key factor in this improvement of clinical outcome has been the development of antiretroviral drugs, which, when taken properly, can decrease viral loads and significantly increase a person's lifespan. While highly active antiretroviral therapy (HAART) can reduce viral loads to undetectable levels in an infected person, allowing the $CD4^+$ T cell population to recover, the drugs are unable to clear the virus, and the patient's immune system remains in a heightened state of activation. Comorbidities associated with chronic inflammation, coupled with the cost of drug regimens, access to care, and the requirement for strict adherence continue to be hurdles to long-term effective drug treatment in the global population (reviewed in (1)).

Given the myriad problems associated with lifelong (and worldwide) HAART treatment, transmission will continue and the development of an HIV-1 vaccine continues to be the most promising solution to fully control the epidemic. Of the three antiviral immune responses, intrinsic, innate, and adaptive, inducing the latter has been the focus of the vaccine research field. Over a period of 28 years with 187 separate HIV vaccine trials (2), we have found that to develop a successful vaccine we must improve our understanding of pathogenesis along with correlates of protection and viral clearance, as well as identify immunogen(s) able to induce such responses.

The invariant feature of CD4 tropism among HIV-1 strains results in the depletion of $CD4^+$ T cells, which, in addition to their own effector functions, are critical for the

help they provide in establishing robust $CD8^+$ T cell and B cell responses (reviewed in (3, 4)). Thus, in almost every case of HIV-1 infection, the immune system is disabled and incapable of producing a controlling adaptive response. Additionally, disruption of T cell homeostasis is disrupted and this, coupled with microbial translocation and persistent exposure to viral antigen, causes a chronic generalized state of immune activation (5). Over time, the immune system collapses and the infected person becomes susceptible to a variety of opportunistic infections. The binding between HIV-1 and CD4, persistent replication, the loss of the target CD4 cell, and immune activation are intertwined in our understanding of pathogenesis. One strategy to address the relationship between loss of CD4 cells and pathogenesis is to dissociate the interaction between HIV-1 and CD4 to observe changes in viral tropism and how this impacts pathogenesis and the host immune system. In such a model, where $CD4^+$ T cells are preserved, would pathogenesis be mitigated and would the immune system be able to control viral replication?

The possibilities of anti-HIV-1 responses resulting from an intact host adaptive immune system are tantalizing and warrant further investigation. We hypothesize that infection in the absence of CD4 targeting will allow host immune responses to be elicited that are qualitatively and/or quantitatively superior to those that occur during pathogenic CD4-tropic infection, resulting in an overall improved clinical outcome. To test this hypothesis, we have derived a novel CD4-independent variant of simian immunodeficiency virus (SIV), characterized its virologic properties in vitro, and evaluated alterations in tropism, pathogenesis, and the host immune response in the rhesus macaque infection model.

Lentiviruses

HIV-1 is a member of the lentivirus genus of retroviruses and as a result of the HIV-1 epidemic lentiviruses have become one of the most researched groups of viruses. As their designation of "*lenti*" (meaning slow) implies, all of these viruses have long incubation periods prior to the appearance of disease. They are further characterized by their ability to persist in the presence of a multifaceted immune response while causing multi-organ disease and high rates of fatality. The genus of lentiviruses can be subdivided into two groups based on their cellular tropism within hosts. Equine infectious anemia virus (EIAV), bovine immunodeficiency virus (BIV) and the small ruminant lentiviruses (SRLVs, including ovine lentiviruses [maedi-visna] and the caprine lentiviruses [CAEV]) are all macrophage-tropic. The feline (FIV) and primate (including simian and human) lentiviruses replicate primarily in lymphocytes, though at least some strains can also replicate in macrophages (6, 7). The reason for this change in tropism between lentiviral groups, which is associated with a change in the number of receptors used for viral entry, has yet to be understood. What is clear, however, are the different disease manifestations of the two viral groups that are a consequence of their differing cell tropisms.

All members of the retrovirus family, including lentiviruses, contain four replicative genes *gag*, *pro*, *pol*, and *env*, which encode the virion structural proteins, protease, reverse transcriptase and integrase, and the surface glycoproteins, respectively. Beyond these four standard retroviral genes, lentiviruses also contain additional small open reading frames (ORFs) located between the *pol* and *env* genes, as well as exons contained within and at the 3' end of the *env* gene that encode for regulatory proteins.

While there is little genetic conservation between the regulatory genes of the different lentiviruses, their functions are highly conserved. The primate lentiviruses contain the highest number of ORFs, including *vif*, *vpu* (*vpx* in HIV-2 and SIV), *vpr*, *tat*, *rev*, and *nef*. The other lentiviruses, FIV, BIV, EIAV, and SRLV typically encode fewer ORFs with orthologs for *rev*, *tat*, and *vif* most commonly conserved (8).

Primate Lentiviruses

Thanks to technological advancements in the last 30 years, we have a clearer understanding of the origins of HIV and the evolutionary history of SIV. While SIV has been circulating in non-human primate populations for at least tens of thousands of years, HIV is a relatively new lentivirus in humans. Currently, natural SIV infection has only been observed in African monkeys and apes, suggesting its emergence after the divergence of the African and Asian Old World monkey lineages, approximately 6-10 million years ago (9, 10). However, it should be noted that incomplete sampling of primate species worldwide for lentiviruses means that we may be underestimating their evolutionary lifespan. Given that SIV has evolved over (at least) tens of thousands of years it is not surprising that many species-specific strains have arisen and infect a wide variety of non-human primates (8, 9, 11). In almost all of these cases the primate species has coevolved with the lentiviral strain to tolerate chronic infection with minimal pathogenic consequences. Emerging evidence suggests that these "natural hosts" of SIV, experience high viral loads, but not the immune activation and disease progression seen with HIV and SIVmac infection. Natural hosts have, through various mechanisms, coevolved with their species-specific SIV strain in an attempt to protect their central

memory $CD4^+$ T cell population (12–16). It is in instances of cross-species transmission of SIV that AIDS-like pathogenesis occurs.

Three cases of cross-species transmission are of special interest in considering the lineage of HIV-1. The first case is of the SIV strain infecting chimpanzees (SIVcpz). Molecular analysis of SIVcpz indicates that this strain is in fact a recombinant virus made up of genes from the SIVs of the red-capped mangabey and of the *Cercopithecus* species (greater spot nosed, mustached, and mona monkeys) (17). This cross-species transmission is likely the result of exposure to infected monkey blood during chimpanzee hunting of other species. While not initially appreciated, this recombinant virus is in fact pathogenic, resulting in increased risk of death, lower birth rates, significant $CD4^+$ T cell loss, and AIDS-like pathologies in chimpanzees (18–20). Not all of the geographically differentiated subspecies of chimpanzee carry SIVcpz, rather it appears that the virus arose in the central subspecies (*P.t. troglodytes*) whose territory overlaps with those of red-capped mangabeys and monkeys of the *Cercopithecus* species, and then later spread to eastern chimpanzees (*P.t. schweinfurthii*) (21, 22). Surprisingly, recent work has also shown cross-species transmission events between central chimpanzees and western lowland gorillas, although it is not yet known whether SIVgor is pathogenic in its host (23, 24).

HIV lineages are also a consequence of cross-species transmission between various monkey and ape species and humans, likely as a result of exposure to infected primate blood or body fluids in the course of hunting for bush meat (25). There are two types of HIV, types 1 and 2, characterized by genetic and pathogenic differences. Within both types there are distinct lineages that are named by letter, and which are themselves

the result of independent cross-species transmissions. Group M of HIV-1 is the pandemic form of HIV, with all other HIV-1 and HIV-2 lineages being far less prevalent as well as geographically restricted. Phylogenic, statistical, and molecular epidemiological analyses of HIV-1 M strains and evolution rates have predicted the last common ancestor of group M occurred around 1910-1930 in colonial west central Africa, specifically in the area of Kinshasa (previously called Leopoldville) (26–30). It is well agreed that cross-species transmissions between non-human primates and humans occurred multiple times over the course of our history; however it is the unfortunate coincidence that HIV-1 group M emerged just as African human populations were transitioning from living in isolated villages to an increasingly urban and mobile lifestyle, thereby increasing the opportunity for human-to-human transmission. As HIV-1 group M spread around the world during the 1900s and early 2000s it further stratified through founder effects into nine subtypes (A-D, F-H, J, K) and more than 40 circulating recombinant forms which are concentrated in geographic regions (31).

The final case of cross-species transmission discussed here occurred accidentally at the California National Primate Research Center (CNPRC) in the 1970s. It is believed that a group of Asian rhesus macaques received tissues from naturally SIV-infected sooty mangabeys in the course of an attempt to develop a non-human primate model for prion disease (32). While the sooty mangabeys were natural hosts of their SIV strain (SIVsmm) and showed no signs of disease, the rhesus macaques experienced an outbreak of lymphoma. This outbreak was seen as an isolated event until the 1980s when researchers at the New England Primate Research Center (NEPRC) isolated the first SIV strain (SIVmac), recognized as the etiologic agent for a transmissible immunodeficiency

characterized by opportunistic infections and tumors (33). The origin of this virus was ultimately traced back to the CNPRC (34). The identification of SIV in the 1980s followed quickly on the discovery of HIV-1, and became the first animal model that could recapitulate the virologic and pathologic properties of HIV-1 in humans.

Initial attempts at infecting monkeys and apes with HIV-1 failed (35), due to then unrecognized host-specific restriction factors (reviewed in (36). Once it was appreciated that Asian macaque lineages were susceptible to the SIV strains of African non-human primates, and that infection resulted in AIDS-like symptoms, SIVsmm-infected Asian macaques began to be used as a non-human primate model of HIV-1 infection. Due to certain differences between SIV and HIV-1, including coreceptor usage (37), diversity of SIV Env variants available (34), and susceptibility to inhibitors of various viral proteins (38), additional effort has been spent on developing HIV-1/SIV chimeric viruses. These chimeric viruses can be classified into two general groups, SIV strains with HIV genes (SHIVs) and HIV-1 strains that are adapted to replicate in macaques (stHIVs) (34, 39). While these viral tools are still under development, they are an important bridge between the SIV-based animal model and the HIV-1 infected human population.

Virion Structure

The basic structure of retroviruses applies to lentiviruses as well: virions are enveloped, approximately 100 nm in diameter, and contain a Gag protein core surrounding two copies of viral RNA. The Gag protein is made as a precursor that is cleaved during virion assembly to give rise to the processed matrix/MA, capsid/CA, and nucleocapsid/NC proteins. The lentivirus core structure is distinguishable from other

retroviruses by its cylindrical shape, formed by the CA protein, with MA lining the inner face of the viral envelope membrane and NC in complex with the RNA (40). While the sizes of lentiviral proteins vary somewhat between viral species, the organization of protein domains and their roles in assembly are similar. The *env* gene of HIV and SIV encodes a 160 kDa protein that is glycosylated by host machinery, then proteolytically cleaved within the Golgi to form a heterodimer of gp120 and gp41 (41–43). These heterodimers of gp120 and gp41 assemble as trimers at the cell surface at sites of budding where gp120 acts as the extracellular cap and gp41 anchors into the membrane (44–47). Wild-type strains of HIV-1 and SIV generally have only 7-10 trimers per virion, though specific mutations, truncation of the gp41 cytoplasmic tail in particular, are known to greatly increase the expression of Env on the virion surface (48, 49).

Within gp120 and gp41 there are distinct regions of amino acids that may differ genetically but serve the same function in the course of entry. Gp120 contains five variable loops (V1-5) and five relatively conserved domains (C1-5). Each of the variable regions adopts a loop structure due to one or more disulfide bonds at the base of the loop. In the unbound trimer these loops lie at the surface with glycans pointing outwards creating a so-called "shield". Gp41 contains a fusion peptide at its N terminus and two helical regions (HR) in the extracellular domain, all three of which are crucial for the final steps of viral entry into the target cell (50, 51).

Many features of the primate lentivirus Env protein structure have rendered it a difficult target for the host humoral response. First, the presence of the glycan shield thwarts the host immune response by presenting a dense pattern of host cell carbohydrates, which the immune response recognizes as self and consequently will not

naturally target. Second, the arrangement of the variable loops at the surface of the protein, which exhibit significant genetic variation between strains and rapid evolution within each host, means that the immune system must target a rapidly evolving antigen and frequently falls behind. Finally, the complex and sequential conformational changes that occur during the entry process (discussed in greater detail below) ensure that the conserved regions of Env, and those critical for coreceptor binding and entry, are only exposed once the primary receptor CD4 is bound and the virion is in close proximity to the cell surface, which limits antibody access to these sites by steric hindrance (50, 52–56).

Viral Entry

Attachment. The entry of HIV and SIV into a cell is a multistep process that requires a series of coordinated protein-protein interactions. The first phase is the attachment of the virion to the cell surface. Attachment can happen nonspecifically, through the interaction of Env with negatively charged cell surface heparin sulfate proteoglycans (57), or specifically via interaction with the attachment factors such as α4β7 (58, 59) or DC-SIGN (60, 61). By either mechanism, the virus is captured close to the cell surface, thereby increasing its chances of interacting with the receptors used for entry. It is important to note that while attachment factors can increase the efficiency of infection, they are not strictly required for viral entry.

CD4 Binding. The first required step of viral infection is the binding of Env to its primary receptor, CD4 (62). CD4 is a member of the immunoglobin superfamily and serves as an enhancer of T-cell receptor mediated signaling. Binding occurs via

conserved sites within gp120, located in the C2, C3, and C4 domains. The key interactions for this binding involve Phe-43 and Arg-59 of CD4 and Asp-368, Glu-370, and Trp-427 of gp120 (63–65). Of note is the occurrence of a salt bridge between Arg-59 of CD4 and Asp-368 in HIV (50). The existence of this salt bridge has not been proven yet in SIV, but given the high sequence conservation within the CD4 binding site and the loss of binding upon mutation of the concurrent Asp residue in SIV (at position 385), it is presumed to be present (66). Upon Env binding to CD4, a series of conformational changes occurs within the Env trimer, including a rearrangement of V1/V2, which results in exposure of V3. Concurrent with the movement of the variable loops, a four-stranded β sheet structure, termed the bridging sheet, forms. Both the V3 loop and the bridging sheet are critical for engagement of a secondary receptor, the coreceptor (63, 66).

Coreceptor Binding. The third step in entry is the engagement of the viral coreceptor, CCR5 or CXCR4 for HIV-1. Both of these 7 transmembrane G-protein coupled chemokine receptors (GPCRs) are expressed on activated $CD4^+$ T cells. Most transmitted/founder HIV-1 strains are CCR5-tropic (R5 viruses), with CXCR4 tropism (X4 viruses) developing later in infection as $CD4^+$ $CCR5^+$ T cells are depleted (67). Conversely, SIV strains are CCR5-tropic with little to no use of CXCR4; however, some natural host strains have evolved the ability to utilize alternative coreceptors as discussed below (68).

Fusion. The final stage of viral entry is the fusion of the viral membrane with the cellular membrane, which is mediated by Env. Binding of the coreceptor induces additional conformational changes, which result in the exposure of the hydrophobic gp41 fusion peptide, which then inserts itself into the cellular membrane. The fusion peptide of

each gp41 subunit in the trimer folds at a hinge region, bringing the two HR domains from each gp41 subunit together to form a six helix bundle (69, 70). The action of this bundle formation pulls the two membranes into close proximity with each other and allows for the formation of a fusion pore through which the virion contents can be delivered into the host cell cytoplasm (71).

It has long been thought that the Env was a rigid structure in the unbound state and that the subsequent transitions through distinct conformational states coincided with large releases of energy resulting in the driving of Env into sequentially lower energy states, which cannot be reversed. Recent work, however, has suggested that the unbound Env trimer is much more dynamic and can sample at least three distinct prefusion conformations. The unbound trimer may exist in the stable ground-state configuration or spontaneously transition to transient CD4-bound or coreceptor-bound states. Different strains may sample each of these states at different rates. Additionally, specific mutations within Env are capable of driving the protein into a "dead-end" state in which one conformation is rigidly adopted and the subsequent conformational changes required for entry cannot be completed (72, 73).

Diversity of Lentiviral Receptors

As described previously, HIV and SIV use a two-receptor system for engagement at the cell surface, with HIV utilizing CD4 and CCR5 and/or CXCR4 and SIV utilizing CD4 and primarily CCR5. However, it is important to note that certain strains of SIV are capable of using alternative coreceptors for entry. The alternative coreceptors shown to support SIV entry include the chemokine receptors CCR2, CCR3, CCR8, CXCR6, as

well as the GPCRs: GPR1, GPR15, and APJ (37, 74–83). While most of the work testing these receptors has occurred in cell culture, some key examples of alternative coreceptor usage in vivo have been reported (78, 83, 84).

As discussed earlier, the other mammalian lentiviruses can be divided into two groups based on the pathologies they cause, the types of cells they infect, and the number of receptors they use for entry. FIV is most similar to the primate lentiviruses in that it causes a decline in the number of $CD4^+$ T cells and an acquired immune deficiency syndrome (AIDS) in cats, albeit at lower rates than HIV-1 causes in humans (85). Like HIV and SIV, FIV utilizes a two receptor system for entry and primarily infects $CD4^+$ T cells and macrophages with expansion of tropism to B cells and $CD8^+$ T cells later in infection (86–88). Whereas the primary receptor for HIV and SIV is CD4, FIV engages CD134, a member of the tumor necrosis factor receptor (TNFR) family, as its primary receptor. After binding to CD134, the FIV Env then binds to the chemokine coreceptor CXCR4, similar to certain strains of HIV-1 (88–90). In cats, CD134 is primarily expressed on $CD4^+$ T cells; however, expression analysis of the human and mouse orthologs suggests the presence of CD134 on activated $CD8^+$ T cells, macrophages, and activated B cells (88, 91–93).

The other group of strictly macrophage-tropic lentiviruses has not received as much attention as HIV, SIV, and FIV, and thus efforts to identify cellular receptors used for viral entry have been varied. In the last ten years the receptor for EIAV was identified as Equine Lentivirus Receptor-1 (ELR1) (94), a protein that is related to TNFR proteins, similar to CD134 in felines. Conclusive data has not yet been published regarding the receptors for BIV or SLRVs. Wright et al. (95) hypothesize that CCR5 could serve as the

receptor for BIV based on their cell culture work showing a reduction in BIV infectivity in the presence of the CCR5 antagonists MIP-1α, MIP-1β, and RANTES. Recent work by Crespo, et al. (96, 97) has suggested that SRLVs utilize the mannose receptor for entry. While the receptors for these viruses have yet to be clearly identified, it is agreed that their primary cell target is the macrophage (6–8, 98). This specific tropism results in clear differences in disease course, the most obvious of which being that these macrophage-tropic strains do not cause immunodeficiency. These strains are most commonly considered "wasting diseases" in which animals can experience fever, lethargy, anorexia, neuropathies, and lymphadenopathy (7).

Given the differing pathological consequences of these two subgroups of lentiviruses, which is directly related to their cellular tropism and method of cellular engagement, research into understanding the relationship between the virus and receptor and the consequences this relationship has for both virus and host will be critical to altering clinical outcomes.

SIV As A Model

The HIV research field was greatly advanced in the 1980's with the discovery that infection of rhesus macaques with an SIV from sooty mangabeys recapitulated the pathogenesis and progression to AIDS observed in humans infected with HIV-1. SIV strains used in Asian monkey models have been passaged through multiple animals and selected for specific pathogenic qualities. The prototypic and most often used strains in macaque research are SIVmac251 and SIVmac239. SIVmac251 was isolated as a swarm in a rhesus macaque at the NEPRC and the original swarm (SIVmac251_1991) has been

characterized as having 0.8% diversity (33, 99). Subsequently the SIVmac251 swarm was passaged through three other rhesus macaques from which the clone SIVmac239 was isolated (34). SIVmac239 is extremely well characterized as resulting in high viral loads (10^6-10^7 copies viral RNA/mL of plasma), causing 50% of infected animals to progress to fatal AIDS within a year (100). This viral clone is extremely neutralization resistant, CCR5-tropic, and CD4-dependent. It is considered a rigorous challenge stock to test vaccine candidates in macaques because of its virulence. While in vitro SIVmac239 does not infect macrophages, in vivo the virus evolves, and infection of macrophages has been observed. In certain cases this expansion of tropism affords the virus the ability to expand into other tissue compartments and can result in pathologies within the lung and brain of macaques. It is the characteristic of macrophage infection that originally identified the handful of CD4-independent viruses previously reported in the literature (101–106). The ability of these viruses to efficiently infect macrophages cell culture and in vivo is directly linked to the brain and lung lesions caused by these viruses.

In 2002 Puffer et al. (107) detailed an in vitro comparison between three macrophage-tropic SIV strains that are also capable of CD4-independent entry. The three independently generated strains, SIVmac316, SIVmac1A11, and SIVmac17E-Fr are all closely related to SIVmac239 genetically, but exhibit significant phenotypic differences associated with their altered tropism.

SIVmac316 was isolated from culture supernatants of alveolar macrophages taken by bronchoalveolar lavage from a rhesus macaque 168 days post infection with SIVmac239. The animal from which the strain was isolated had giant cell pneumonia and granulomatous encephalitis (108); both lesions are associated with infection of tissue

macrophages. In vitro evaluation of the SIVmac316 strain revealed it replicated quite well in primary and cultured macrophages, and that this tropism is driven by genetic determinants within *env*, as cloning of the SIVmac316 *env* into the SIVmac239 backbone recapitulated the phenotype of SIVmac316 (108). Strikingly, while SIVmac316 was isolated from macrophage supernatants, when SIVmac316 was inoculated into rhesus macaques, infected macrophages and macrophage-associated pathologies were rarely observed (109). Thus, the SIVmac316 model serves as a cautionary tale of discrepancies between in vitro infection assays and in vivo tropism.

SIVmac1A11 was isolated from the same rhesus macaque that was the origin of the SIVmac251 clone. The SIVmac1A11 strain replicates efficiently in RhPBMCs, human T cell lines, and in both rhesus monocyte-derived macrophages (MDM) and alveolar macrophages (102, 103). While the SIVmac1A11 clone differed from SIVmac239 in multiple genes, the Env protein by itself has been shown to capable of CD4-independent fusion and thus is likely the driver of macrophage tropism (101, 107). Rhesus macaques infected with SIVmac1A11 display transient viremia and do not progress to AIDS (103).

The 17E-Fr SIVmac strain is a chimeric virus made up of the entire *env* and *nef* genes and the 3' LTR of the neurovirulent SIV/17E-Br strain in the backbone of SIVmac239. SIV/17E-Br is an uncloned swarm from SIVmac239 infected rhesus macaques exhibiting CNS disease (110). SIV/17E-Fr retained the macrophage tropism and neurovirulence of SIV/17E-Br but was attenuated in vivo (104). Similar to SIVmac1A11, while multiple genes in SIVmac17E-Fr differ from SIVmac239, the SIVmac17E-Fr Env alone has displayed an ability for CD4-independent fusion (107).

Currently, SIVmac17E-Fr is used in a co-infection model with the immunosuppressive swarm SIV/DeltaB670 to create accelerated CNS disease in pigtail macaques (111).

The 2002 study by Puffer et al. (107) clearly demonstrated that Envs from these three macrophage-tropic SIV strains were capable of CD4-independent cell-cell fusion and pseudotype infection of cells transfected with RhCCR5 but not CD4. Along with this altered cellular tropism and ability to infect cells without CD4, these Envs were also distinctive for their neutralization sensitivity to both sera from SIVmac239-infected animals and monoclonal antibodies. The attenuation and neutralization sensitivity of these strains likely explain why they arise infrequently in vivo, and when they do why they often replicate primarily in immune privileged sites like the brain rather than systemically.

Ortiz, et al. (112), published another key study in the field of CD4-independent SIVs in 2011, in which rhesus macaques were administered an anti-CD4 antibody, resulting in the depletion of peripheral $CD4^+$ T cells. These animals were then infected intravenously with the SIVmac251 swarm. Animals that had been depleted of CD4 T cells exhibited high and sustained viral loads with an accelerated progression to fatal AIDS in comparison to the SIVmac251-infected undepleted control animals. Analysis of *env* clones isolated during infection revealed that the majority of Envs from depleted animals were capable of CD4-independent infection in a pseudotype assay while all Env proteins from undepleted animals were strictly CD4-dependent. It is important to note that the isolated Envs capable of CD4-independent pseudotype infection did so at levels approximately 40-60% of infection on cells with CD4 and CCR5. These *envs* arose by 42 days post infection and appear to have originated from a macrophage-tropic variant

contained within the inoculum swarm (it either existed in the inoculum or rapidly evolved from a closely related variant) that quickly dominated the viral swarm in the depleted animals where $CD4^+$ T cells were scarce (112). Similar to the viral strains described above, animals that had been depleted of $CD4^+$ T cells and had developed macrophage-tropic strains exhibited higher rates of lung and brain disease (112, 113)(M. Paiaridini, personal communication).

Recent work describing a novel macrophage tropic strain isolated from the blood of an SIVmac251-infected rhesus macaque noted that CD4-independent transmission occurred only in the context of cell-cell contact and not under cell-free conditions (114, 115). The macrophage tropic phenotype of this strain was attributed to the loss of a single glycosylation site in the V2 region of Env (114). When this mutation alone was introduced into SIVmac239, it resulted in an increase in macrophage infection in vitro and CD4-independent cell-cell fusion, but not infection of a CD4-negative cell line (115). The authors posit that CD4-independent cell-cell transmission may be an important mechanism for promoting macrophage tropism within tissues, as this mode of transmission has been shown to be more efficient than cell-free virus infection and to protect viruses from inhibition by neutralizing antibodies (115–118).

It is important to note that while some macrophage-tropic Envs have been shown to be CD4-independent in vitro, it is not clear whether these strains infect in a CD4-independent manner in vivo. Presumably, if CD4 is present along with CCR5 on the surface of cell, the virus will utilize both receptors to increase viral entry efficiency. Additionally, many of the "CD4-independent" SIV Envs studied in vitro have contained a premature stop codon in the cytoplasmic tail, as a result of passaging through human T

cell lines (119). One consequence of this truncation is the increase of Env expression on virions (48), thereby potentially increasing Env-receptor fusion frequency. Infectious molecular clones of many of these "CD4i" viruses with full length cytoplasmic tails have yet to be tested for their ability to infect $CD4^-$ $CCR5^+$ cell lines and primary cells, and whether infection occurs as efficiently as when CD4 is present. Thus, while macrophage-tropic SIV strains that are capable of CD4-independent entry have been well characterized in vitro, a true CD4-independent variant, i.e. one that does not bind CD4, has not previously been evaluated in vivo.

Goals of This Thesis

As discussed above, while macrophage tropic strains with CD4-independent properties have been characterized, a truly independent variant, i.e. one with absent or greatly reduced CD4 binding, has yet to be tested in an in vivo model. Here I present the development and testing of a novel, CD4-independent strain of SIV in vivo. In Chapter 2 I describe the derivation and virologic characteristics of a CD4-independent SIVmac239 variant, documenting the mutations that conferred CD4-independence, the effects of these mutations on neutralization sensitivity, and alterations in cell tropism in vitro. I then, in Chapter 3, examine the effect of CD4-independence on viral replication, pathogenesis, viral evolution, and host immune responses in rhesus macaques, as well as the results of a challenge study. Finally, in Chapter 4 I will provide a summary of our work and suggest future avenues of exploration.

References

1. **Piot P**, **Abdool Karim SS**, **Hecht R**, **Legido-Quigley H**, **Buse K**, **Stover J**, **Resch S**, **Ryckman T**, **Møgedal S**, **Dybul M**, **Goosby E**, **Watts C**, **Kilonzo N**, **McManus J**, **Sidibé M**. 2015. Lancet Commission on Defeating AIDS—Advancing Global Health. Lancet **386**:171–218.

2. **O'Connell RJ**, **Kim JH**, **Corey L**, **Michael NL**. 2012. Human Immunodeficiency Virus Vaccine Trials, p. 483–504. *In* HIV From Biology to Prevention and Treatment.

3. **Wiesel M**, **Oxenius A**. 2012. From crucial to negligible: Functional CD8+ T-cell responses and their dependence on CD4+T-cell help. Eur J Immunol **42**:1080–1088.

4. **Crotty S**. 2015. A brief history of T cell help to B cells. Nat Rev Immunol **15**:185–9.

5. **Younas M**, **Psomas C**, **Reynes J**, **Corbeau P**. 2015. Immune activation in the course of HIV-1 infection: Causes, phenotypes and persistence under therapy. Rev in Antiretro Res: doi: 10.1111/hiv.12310.

6. **Clements JE**, **Christine Zink M**. 1996. Molecular biology and pathogenesis of animal lentivirus infections. Clin Microbiol Rev **9**:100–117.

7. **St-Louis M-C**, **Cojocariu M**, **Archambault D**. 2004. The molecular biology of bovine immunodeficiency virus: a comparison with other lentiviruses. Anim Health Res Rev **5**:125–143.

8. **Gifford RJ**. 2012. Viral evolution in deep time: Lentiviruses and mammals. Trends Genet **28**:89–100.

9. **Apetrei C**, **Robertson DL**, **Marx PA**. 2004. The history of SIVs and AIDS: epidemiology, phylogeny and biology of isolates from naturally SIV infected non-human primates (NHP) in Africa. Front Biosci **9**:225–254.

10. **Fabre PH**, **Rodrigues A**, **Douzery EJP**. 2009. Patterns of macroevolution among primates inferred from a supermatrix of mitochondrial and nuclear DNA. Mol Phylogenet Evol **53**:808–825.

11. **Worobey M**, **Telfer P**, **Souquière S**, **Hunter M**, **Coleman CA**, **Metzger MJ**, **Reed P**, **Makuwa M**, **Hearn G**, **Honarvar S**, **Roques P**, **Apetrei C**, **Kazanji M**, **Marx PA**. 2010. Island biogeography reveals the deep history of SIV. Science **329**:1487.

12. **Gordon SN**, **Dunham RM**, **Engram JC**, **Estes J**, **Wang Z**, **Klatt NR**, **Paiardini M**, **Pandrea I V**, **Apetrei C**, **Sodora DL**, **Lee HY**, **Haase AT**, **Miller MD**, **Kaur A**, **Staprans SI**, **Perelson AS**, **Feinberg MB**, **Silvestri G**. 2008. Short-lived infected cells support virus replication in sooty mangabeys naturally infected with simian immunodeficiency virus: implications for AIDS pathogenesis. J Virol **82**:3725–3735.

13. **Pandrea I**, **Apetrei C**, **Gordon S**, **Barbercheck J**, **Dufour J**, **Bohm R**, **Sumpter B**, **Roques P**, **Marx PA**, **Hirsch VM**, **Kaur A**, **Lackner AA**, **Veazey RS**, **Silvestri G**. 2008. Paucity of CD4 + CCR5 + T cells is a typical feature of natural SIV hosts. Blood **109**:1069–1076.

14. **Paiardini M**, **Pandrea I**, **Apetrei C**, **Silvestri G**. 2009. Lessons learned from the natural hosts of HIV-related viruses. Annu Rev Med **60**:485–495.

15. **Chahroudi A**, **Bosinger SE**, **Vanderford TH**, **Paiardini M**, **Silvestri G**. 2012. Natural SIV Hosts: Showing AIDS the Door. Science **335**:1188–1193.

16. **Perkins MR**, **Briant JA**, **Calantone N**, **Whitted S**, **Vinton CL**, **Klatt NR**, **Ourmanov I**, **Ortiz AM**, **Hirsch VM**, **Brenchley JM**. 2014. Homeostatic Cytokines Induce CD4 Downregulation in African Green Monkeys Independently of Antigen Exposure To Generate Simian Immunodeficiency Virus-Resistant CD8 T Cells. J Virol **88**:10714–10724.

17. **Bailes E**, **Gao F**, **Bibollet-Ruche F**, **Courgnaud V**, **Peeters M**, **Marx PA**, **Hahn BH**, **Sharp PM**. 2003. Hybrid origin of SIV in chimpanzees. Science **300**:1713.

18. **Keele BF**, **Jones JH**, **Terio KA**, **Estes JD**, **Rebecca S**, **Wilson ML**, **Li Y**, **Learn GH**, **Beasley TM**, **Wroblewski E**, **Mosser A**, **Raphael J**, **Lonsdorf E V**, **Travis DA**, **Mlengeya T**, **Michael J**, **Else JG**, **Silvestri G**, **Goodall J**, **Sharp PM**, **George M**, **Pusey AE**, **Hahn BH**. 2009. Increased Mortality and AIDS-like Immunopathology in Wild Chimpanzees infected with SIVcpz. Nature **460**:515–519.

19. **Rudicell RS**, **Jones JH**, **Wroblewski EE**, **Learn GH**, **Li Y**, **Robertson JD**, **Greengrass E**, **Grossmann F**, **Kamenya S**, **Pintea L**, **Mjungu DC**, **Lonsdorf E V**, **Mosser A**, **Lehman C**, **Collins DA**, **Keele BF**, **Goodall J**, **Hahn BH**, **Pusey AE**, **Wilson ML**. 2010. Impact of simian immunodeficiency virus infection on chimpanzee population dynamics. PLoS Pathog **6**(9): e1001116. doi:10.1371/journal.ppat.1001116.

20. **Terio KA**, **Kinsel MJ**, **Raphael J**, **Mlengeya T**, **Lipende I**, **Kirchhoff CA**, **Gilagiza B**, **Wilson ML**, **Kamenya S**, **Estes JD**, **Keele BF**, **Rudicell RS**, **Liu W**, **Patton S**, **Collins A**, **Hahn BH**, **Travis DA**, **Lonsdorf EV.** 2011. Pathologic Lesions in Chimpanzees (Pan Trogylodytes Schweinfurthii) From Gombe National Park, Tanzania, 2004-2010. J Zoo Wildl Med **42**:597–607.

21. **Sharp P**, **Shaw G**, **Hahn B**. 2005. Simian Immunodeficiency Virus Infection of Chimpanzees. J Virol **79**:3891–3902.

22. **Keele BF**, **Van Heuverswyn F**, **Li Y**, **Bailes E**, **Takehisa J**, **Santiago ML**, **Bibollet-Ruche F**, **Chen Y**, **Wain LV**, **Liegeois F**, **Loul S**, **Ngole EM**, **Bienvenue Y**, **Delaporte E**, **Brookfield JFY**, **Sharp PM**, **Shaw GM**, **Peeters M**, **Hahn BH**. 2006. Chimpanzee Reservoirs of Pandemic and Nonpandemic HIV-1. Science **313**:523–526.

23. **Van Heuverswyn F**, **Li Y**, **Neel C**, **Bailes E**, **Keele BF**, **Liu W**, **Loul S**, **Butel C**, **Liegeois F**, **Bienvenue Y**, **Ngolle EM**, **Sharp PM**, **Shaw GM**, **Delaporte E**, **Hahn BH**, **Peeters M**. 2006. Human immunodeficiency viruses: SIV infection in wild gorillas. Nature **444**:164.

24. **Takehisa J**, **Kraus MH**, **Ayouba A**, **Bailes E**, **Van Heuverswyn F**, **Decker JM**, **Li Y**, **Rudicell RS**, **Learn GH**, **Neel C**, **Ngole EM**, **Shaw GM**, **Peeters M**, **Sharp PM**, **Hahn BH**. 2009. Origin and biology of simian immunodeficiency virus in wild-living western gorillas. J Virol **83**:1635–1648.

25. **Peeters M**, **Courgnaud V**, **Abela B**, **Auzel P**, **Pourrut X**, **Bibollet-Ruche F**, **Loul S**, **Liegeois F**, **Butel C**, **Koulagna D**, **Mpoudi-Ngole E**, **Shaw GM**, **Hahn BH**, **Delaporte E**. 2002. Risk to human health from a plethora of Simian immunodeficiency viruses in primate bushmeat. Emerg Infect Dis **8**:451–457.

26. **Zhu T**, **Korber BT**, **Nahmias AJ**, **Hooper E**, **Sharp PM**, **Ho DD**. 1998. An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. Nature **391**:594–597.

27. **Korber B**, **Muldoon M**, **Theiler J**, **Gao F**, **Gupta R**, **Lapedes A**, **Hahn BH**, **Wolinsky S**, **Bhattacharya T**. 2000. Timing the ancestor of the HIV-1 pandemic strains. Science **288**:1789–1796.

28. **Vidal N**, **Peeters M**, **Mulanga-Kabeya C**, **Nzilambi N**, **Robertson D**, **Ilunga W**, **Sema H**, **Tshimanga K**, **Bongo B**, **Delaporte E**. 2000. Unprecedented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. J Virol **74**:10498–10507.

29. **Worobey M**, **Gemmel M**, **Teuwen DE**, **Haselkorn T**, **Bunce M**, **Muyembe J**, **Kabongo JM**, **Kalengayi M**, **Marck E Van**, **Gilbert MTP**, **Wolinsky SM**. 2008. Direct Evidence of Extensive Diversity of HIV-1 in Kinshasa by 1960. Nature **455**:661–664.

30. **Sharp PM**, **Hahn BH**. 2008. AIDS: prehistory of HIV-1. Nature **455**:605–606.

31. **Taylor BS**, **Hammer SM**. 2008. The challenge of HIV-1 subtype diversity. N Engl J Med **359**:1965–1966.

32. **Apetrei C**, **Lerche NW**, **Pandrea I**, **Gormus B**, **Silvestri G**, **Kaur A**, **Robertson DL**, **Hardcastle J**, **Lackner AA**, **Marx PA**. 2006. Kuru experiments triggered the emergence of pathogenic SIVmac. AIDS **20**:317–321.

33. **Daniel MD**, **Letvin NL**, **King NW**, **Kannagi M**, **Sehgal PK**, **Hunt RD**, **Essex M**, **Desrosiers RC**. 1985. Isolation of T-Cell Tropic HTLV-III-Like Retrovirus from Macaques. Science **228**:1201–1204.

34. **Hatziioannou T**, **Evans DT**. 2012. Animal models for HIV/AIDS research. Nat Rev Microbiol **10**:852–867.

35. **Morrow WJ**, **Homsy J**, **Eichberg JW**, **Krowka J**, **Pan LZ**, **Gaston I**, **Legg H**, **Lerche N**, **Thomas J**, **Levy JA**. 1989. Long-term observation of baboons, rhesus monkeys, and chimpanzees inoculated with HIV and given periodic immunosuppressive treatment. AIDS Res Hum Retroviruses **5**:233–45.

36. **Simon V**, **Bloch N**, **Landau NR**. 2015. Intrinsic host restrictions to HIV-1 and mechanisms of viral escape. Nat Immunol **16**:546–553.

37. **Sina ST**, **Ren W**, **Cheng-Mayer C**. 2011. Coreceptor use in nonhuman primate models of HIV infection. J Transl Med **9**(Supp 1):S7.

38. **Balzarini J**, **Weeger M**, **Camarasa M-J**, **De Clerq E**, **Uberla K**. 1995. Sensitivity/resistance profile of a Simian Immunodeficiency Virus containing the reverse transcriptase gene of Human Immunodeficiency Type 1 (HIV-1) toward the HIV-1-specific non-nucleoside reverse transcriptase inhibitors. Biochem Biophys Res Commun **211**:850–856.

39. **Hatziioannou T**, **Ambrose Z**, **Chung NPY**, **Piatak M**, **Yuan F**, **Trubey CM**, **Coalter V**, **Kiser R**, **Schneider D**, **Smedley J**, **Pung R**, **Gathuka M**, **Estes JD**, **Veazey RS**, **KewalRamani VN**, **Lifson JD**, **Bieniasz PD**. 2009. A macaque model of HIV-1 infection. Proc Natl Acad Sci **106**:4425–4429.

40. **Campbell EM**, **Hope TJ**. 2015. HIV-1 capsid: the multifaceted key player in HIV-1 infection. Nat Rev Microbiol **13**:471–483.

41. **Allan AJS**, **Coligan JE**, **Barin F**, **Mclane MF**, **Sodroski JG**, **Rosen CA**, **Lee TH**, **Essex M**. 1985. Major Glycoprotein Antigens that Induce Antibodies in AIDS Patients are Encoded by HTLV-III. Science **228**:1091–1094.

42. **Veronese FD**, **Devico AL**, **Copeland TD**, **Oroszlan S**, **Gallo RC**, **Sarngadharan MG**. 1985. Characterization of gp41 as the Transmembrane Protein Coded by the HTLV-III/LAV Envelope Gene. Science **229**:1402–1405.

43. **Willey RL**, **Bonifacino JS**, **Potts BJ**, **Martin MA**, **Klausner RD**. 1988. Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. Proc Natl Acad Sci **85**:9580–9584.

44. **Earl PL**, **Doms RW**, **Moss B**. 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. Proc Natl Acad Sci **87**:648–652.

45. **Pinter A**, **Honnen WJ**, **Tilley SA**, **Bona C**, **Zaghouani H**, **Gorny MK**, **Zolla-Pazner S**. 1989. Oligomeric structure of gp41, the transmembrane protein of human immunodeficiency virus type 1. J Virol **63**:2674–2679.

46. **Weiss CD**, **Levy JA**, **White JM**. 1990. Oligomeric organization of gp120 on infectious human immunodeficiency virus type 1 particles. J Virol **64**:5674–5677.

47. **Kowalski M**, **Potz J**, **Basiripour L**, **Dorfman T**, **Goh WC**, **Terwilliger E**, **Dayton A**, **Rosen C**, **Haseltine W**, **Sodroski J**. 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science **237**:1351–1355.

48. **Zhu P**, **Chertova E**, **Bess J**, **Lifson JD**, **Arthur LO**, **Liu J**, **Taylor KA**, **Roux KH**. 2003. Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions. Proc Natl Acad Sci **100**:15812–15817.

49. **Zhu P**, **Liu J**, **Bess J**, **Chertova E**, **Lifson JD**, **Grisé H**, **Ofek GA**, **Taylor KA**, **Roux KH**. 2006. Distribution and three-dimensional structure of AIDS virus envelope spikes. Nature **441**:847–852.

50. **Wyatt R**, **Sodroski J**. 1998. The HIV-1 Envelope Glycoproteins: Fusogens, Antigens, and Immunogens. Science **280**:1884–1888.

51. **Wyatt R**, **Kwong PD**, **Desjardins E**, **Sweet RW**, **Robinson J**, **Hendrickson WA**, **Sodroski JG**. 1998. The Antigenic Structure of the HIV gp120 Envelope Glycoprotein. Nature **393**:705–711.

52. **Labrijn AF**, **Poignard P**, **Raja A**, **Zwick MB**, **Delgado K**, **Franti M**, **Binley J**, **Vivona V**, **Grundner C**, **Huang C-C**, **Venturi M**, **Petropoulos CJ**, **Wrin T**, **Dimitrov DS**, **Robinson J**, **Kwong PD**, **Wyatt RT**, **Sodroski J**, **Burton DR**. 2003. Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. J Virol **77**:10557–10565.

53. **Gershoni JM**, **Denisova G**, **Raviv D**, **Smorodinsky NI**, **Buyaner D**. 1993. HIV binding to its receptor creates specific epitopes for the CD4/gp120 complex. FASEB J **7**:1185–1187.

54. **Sattentau QJ**, **Moore JP**. 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J Exp Med **174**:407–415.

55. **Thali M**, **Moore JP**, **Furman C**, **Charles M**, **Ho DD**, **Robinson J**, **Sodroski J**. 1993. Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. J Virol **67**:3978–3988.

56. **Wyatt R**, **Moore J**, **Accola M**, **Desjardin E**, **Robinson J**, **Sodroski J**. 1995. Involvement of the V1/V2 variable loop structure in the exposure of human immunodeficiency virus type 1 gp120 epitopes induced by receptor binding. J Virol **69**:5723–5733.

57. **Saphire ACS**, **Bobardt MD**, **Zhang Z**, **Gallay PA**, **Zhang ZHE**, **David G**. 2001. Syndecans Serve as Attachment Receptors for Human Immunodeficiency Virus Type 1 on Macrophages Syndecans Serve as Attachment Receptors for Human Immunodeficiency Virus Type 1 on Macrophages. J Virol **75**:9187–9200.

58. **Arthos J**, **Cicala C**, **Martinelli E**, **Macleod K**, **Van Ryk D**, **Wei D**, **Xiao Z**, **Veenstra TD**, **Conrad TP**, **Lempicki RA**, **McLaughlin S**, **Pascuccio M**, **Gopaul R**, **McNally J**, **Cruz CC**, **Censoplano N**, **Chung E**, **Reitano KN**, **Kottilil S**, **Goode DJ**, **Fauci AS**. 2008. HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells. Nat Immunol **9**:301–309.

59. **Cicala C**, **Martinelli E**, **McNally JP**, **Goode DJ**, **Gopaul R**, **Hiatt J**, **Jelicic K**, **Kottilil S**, **Macleod K**, **O'Shea A**, **Patel N**, **Van Ryk D**, **Wei D**, **Pascuccio M**, **Yi L**, **McKinnon L**, **Izulla P**, **Kimani J**, **Kaul R**, **Fauci AS**, **Arthos J**. 2009. The integrin alpha4beta7 forms a complex with cell-surface CD4 and defines a T-cell subset that is highly susceptible to infection by HIV-1. Proc Natl Acad Sci **106**:20877–20882.

60. **Geijtenbeek TB**, **Kwon DS**, **Torensma R**, **van Vliet SJ**, **van Duijnhoven GC**, **Middel J**, **Cornelissen IL**, **Nottet HS**, **Kewal-Ramani VN**, **Littman DR**, **Figdor CG**, **van Kooyk Y**. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell **100**:587–597.

61. **Ugolini S**, **Mondor I**, **Sattentau QJ**. 1999. HIV-1 attachment: Another look. Trends Microbiol **7**:144–149.

62. **Maddon PJ**, **Dalgleish AG**, **McDougal JS**, **Clapham PR**, **Weiss RA**, **Axel R**. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47**:333–348.

63. **Kwong PD**, **Wyatt R**, **Robinson J**, **Sweet RW**, **Sodroski J**, **Hendrickson WA**. 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393**:648–59.

64. **Arthos J**, **Deen KC**, **Chaikin IM**, **Fornwald JA**, **Sathe G**, **Sattentau QJ**,

Clapham PR, **Weiss RA**, **McDougal JS**, **Pietropaolo C**. 1989. Identification of the residues in human CD4 critical for the binding of HIV. Cell **57**:469–481.

65. **Wu H**, **Myszka DG**, **Tendian SW**, **Brouillette CG**, **Sweet RW**, **Chaiken IM**, **Hendrickson WA**. 1996. Kinetic and structural analysis of mutant CD4 receptors that are defective in HIV gp120 binding. Proc Natl Acad Sci **93**:15030–5.

66. **Chen B**, **Vogan EM**, **Gong H**, **Skehel JJ**, **Wiley DC**, **Harrison SC**. 2005. Structure of an unliganded simian immunodeficiency virus gp120 core. Nature **433**:834–841.

67. **Connor RI**, **Sheridan KE**, **Ceradini D**, **Choe S**, **Landau NR**. 1997. Change in coreceptor use correlates with disease progression in HIV-1 infected individuals. J Exp Med **185**:621–628.

68. **Chen Z**, **Gettie A**, **Ho DD**, **Marx PA**. 1998. Primary SIVsm isolates use the CCR5 coreceptor from sooty mangabeys naturally infected in west Africa: a comparison of coreceptor usage of primary SIVsm, HIV-2, and SIVmac. Virology **246**:113–124.

69. **Chan DC**, **Fass D**, **Berger JM**, **Kim PS**. 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell **89**:263–273.

70. **Weissenhorn W**, **Dessen A**, **Harrison SC**, **Skehel JJ**, **Wiley DC**. 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature **387**:426-430.

71. **Melikyan GB**. 2008. Common principles and intermediates of viral protein-mediated fusion: the HIV-1 paradigm. Retrovirology **5**: doi:10.1186?1742-4690-5-111.

72. **Munro JB**, **Gorman J**, **Ma X**, **Zhou Z**, **Arthos J**, **Burton DR**, **Koff WC**, **Courter JR**, **Smith III, AB**, **Kwong PD**, **Blanchard SC**, **Mothes W**. 2014. Conformational dynamics of single HIV-1 envelope trimers on the surface of native virions. Science **346**:759–763.

73. **Kwon YD**, **Finzi A**, **Wu X**, **Dogo-Isonagie C**, **Lee LK**, **Moore LR**, **Schmidt SD**, **Stuckey J**, **Yang Y**, **Zhou T**, **Zhu J**, **Vicic DA**, **Debnath AK**, **Shapiro L**, **Bewley CA**, **Mascola JR**, **Sodroski JG**, **Kwong PD**. 2012. Unliganded HIV-1 gp120 core structures assume the CD4-bound conformation with regulation by quaternary interactions and variable loops. Proc Natl Acad Sci **109**:5663–5668.

74. **Deng HK**, **Unutmaz D**, **Kewal-Ramani VN**, **Littman DR**. 1997. Expression cloning of new receptors used by simian and human immunodeficiency viruses. Nature **388**:296–300.

75. **Farzan M**, **Choe H**, **Martin K**, **Marcon L**, **Hofmann W**, **Karlsson G**, **Sun Y**, **Barrett P**, **Marchand N**, **Sullivan N**, **Gerard N**, **Gerard C**, **Sodroski J**. 1997. Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection. J Exp Med **186**:405–411.

76. **Edinger AL**, **Hoffman TL**, **Sharron M**, **Lee B**, **Dowd BO**, **Doms RW**. 1998. Use of GPR1 , GPR15 , and STRL33 as Coreceptors by Diverse Human Immunodeficiency Virus Type 1 and Simian Immunodeficiency Virus Envelope Proteins. Pathology **249**:367–378.

77. **Edinger AL**, **Hoffman TL**, **Sharron M**, **Lee B**, **Yi Y**, **Choe W**, **Kolson DL**, **Mitrovic B**, **Zhou Y**, **Faulds D**, **Collman RG**, **Hesselgesser J**, **Horuk R**, **Doms RW**. 1998. An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus. J Virol **72**:7934–7940.

78. **Elliott STC**, **Wetzel KS**, **Francella N**, **Bryan S**, **Romero DC**, **Riddick NE**, **Shaheen F**, **Vanderford T**, **Derdeyn CA**, **Silvestri G**, **Paiardini M**, **Collman RG**. 2015. Dualtropic CXCR6/CCR5 Simian Immunodeficiency Virus (SIV) Infection of Sooty Mangabey Primary Lymphocytes: Distinct Coreceptor Use in Natural versus Pathogenic Hosts of SIV. J Virol **89**:9252–9261.

79. **Yi Y**, **Singh A**, **Isaacs SN**, **Collman RG**. 2001. A CCR5/CXCR4-independent coreceptor pathway on human macrophages supports efficient SIV env-mediated fusion but not infection: implications for alternative pathways of viral entry. Virology **284**:142–51.

80. **Puffer BA**, **Sharron M**, **Coughlan CM**, **Baribaud F**, **McManus CM**, **Lee B**, **David J**, **Price K**, **Horuk R**, **Tsang M**, **Doms RW**. 2000. Expression and coreceptor function of APJ for primate immunodeficiency viruses. Virology **276**:435–44.

81. **Edinger AL**, **Clements JE**, **Doms RW**. 1999. Chemokine and orphan receptors in HIV-2 and SIV tropism and pathogenesis. Virology **260**:211–21.

82. **Rucker J**, **Edinger AL**, **Sharron M**, **Samson M**, **Lee B**, **Berson JF**, **Yi Y**, **Margulies B**, **Collman RG**, **Doranz BJ**, **Parmentier M**, **Doms RW**. 1997. Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses. J Virol **71**:8999–9007.

83. **Gautam R**, **Gaufin T**, **Butler I**, **Gautam A**, **Barnes M**, **Mandell D**, **Pattison M**, **Tatum C**, **Macfarland J**, **Monjure C**, **Marx PA**, **Pandrea I**, **Apetrei C**. 2009. Simian immunodeficiency virus SIVrcm, a unique CCR2-tropic virus, selectively depletes memory CD4+ T cells in pigtailed macaques through expanded coreceptor usage in vivo. J Virol **83**:7894–7908.

84. **Riddick NE**, **Hermann EA**, **Loftin LM**, **Elliott ST**, **Wey WC**, **Cervasi B**, **Taaffe J**, **Engram JC**, **Li B**, **Else JG**, **Li Y**, **Hahn BH**, **Derdeyn CA, Sodora DL**, **Apetrei C**, **Paiardini M**, **Silvestri G**, **Collman RG**. 2010. A Novel CCR5 Mutation Common in Sooty Mangabeys Reveals SIVsmm Infection of CCR5-Null Natural Hosts and Efficient Alternative Coreceptor Use In Vivo. PLoS Pathog **6**(8): e1001064. doi:10.1371/journal.ppat.1001064.

85. **Taniwaki SA**, **Figueiredo AS**, **Araujo JP**. 2013. Virus-host interaction in feline immunodeficiency virus (FIV) infection. Comp Immunol Microbiol Infect Dis **36**:549–557.

86. **English R V**, **Johnson CM**, **Gebhard DH**, **Tompkins MB**. 1993. In vivo lymphocyte tropism of feline immunodeficiency virus. J Virol **67**:5175–5186.

87. **Dean GA**, **Reubel GH**, **Moore PF**, **Pedersen NC**. 1996. Proviral burden and infection kinetics of feline immunodeficiency virus in lymphocyte subsets of blood and lymph node. J Virol **70**:5165–5169.

88. **Shimojima M**, **Miyazawa T**, **Ikeda Y**, **McMonagle EL**, **Haining H**, **Akashi H**, **Takeuchi Y**, **Hosie MJ**, **Willett BJ**. 2004. Use of CD134 As a Primary Immunodeficiency Virus. Science **303**:1192–1195.

89. **Willett BJ**, **Hosie MJ**. 2008. Chemokine receptors and co-stimulatory molecules: Unravelling feline immunodeficiency virus infection. Vet Immunol Immunopathol **123**:56–64.

90. **Willett BJ**, **Hosie MJ**. 2013. The virus-receptor interaction in the replication of feline immunodeficiency virus (FIV). Curr Opin Virol **3**:670–675.

91. **Stüber E**, **Strober W**. 1996. The T cell-B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response. J Exp Med **183**:979–989.

92. **Gramaglia I**, **Weinberg AD**, **Lemon M**, **Croft M**. 1998. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. J Immunol **161**:6510–6517.

93. **Baum PR**, **Gayle RB**, **Ramsdell F**, **Srinivasan S**, **Sorensen RA**, **Watson ML**, **Seldin MF**, **Baker E**, **Sutherland GR**, **Clifford KN**. 1994. Molecular characterization of murine and human OX40/OX40 ligand systems: identification of a human OX40 ligand as the HTLV-1-regulated protein gp34. EMBO J **13**:3992–4001.

94. **Zhang B**, **Jin S**, **Jin J**, **Li F**, **Montelaro RC**. 2005. A tumor necrosis factor receptor family protein serves as a cellular receptor for the macrophage-tropic equine lentivirus. Proc Natl Acad Sci **102**:9918–9923.

95. **Wright SM**, **Mleczko A**, **Coats KS**. 2002. Bovine immunodeficiency virus expression in vitro is reduced in the presence of beta-chemokines, MIP-1alpha, MIP-1beta and RANTES. Vet Res Commun **26**:239–250.

96. **Crespo H**, **Reina R**, **Glaria I**, **Ramírez H**, **De Andrés X**, **Jáuregui P**, **Luján L**, **Martínez-Pomares L**, **Amorena B**, **De Andrés DF**. 2011. Identification of the ovine mannose receptor and its possible role in Visna/Maedi virus infection. Vet Res **42**:28.

97. **Crespo H**, **Jauregui P**, **Glaria I**, **Sanjosé L**, **Polledo L**, **García-Marín JF**, **Luján L**, **De Andrés D**, **Amorena B**, **Reina R**. 2012. Mannose receptor may be involved in small ruminant lentivirus pathogenesis. Vet Res **43**:1–6.

98. **Forsman A**, **Weiss RA**. 2008. Why is HIV a pathogen? Trends Microbiol **16**:555–60.

99. **Strickland SL**, **Gray RR**, **Lamers SL**, **Burdo TH**, **Huenink E**, **Nolan DJ**, **Nowlin B**, **Alvarez X**, **Midkiff CC**, **Goodenow MM**, **Williams K**, **Salemi M**. 2011. Significant Genetic Heterogeneity of the SIVmac251 Viral Swarm Derived from Different Sources. AIDS Res Hum Retroviruses **27**:1327–1332.

100. **Kestler H**, **Kodama T**, **Ringler D**, **Marthas M**, **Pedersen N**, **Lackner AA**,

Regier D, **Sehgal P**, **Daniel M**, **King N**. 1990. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science **248**:1109–12.

101. **Banapour B**, **Marthas ML**, **Ramos RA**, **Lohman BL**, **Unger RE**, **Gardner MB**, **Pedersen NC**, **Luciw PA**. 1991. Identification of viral determinants of macrophage tropism for simian immunodeficiency virus SIVmac. J Virol **65**:5798–5805.

102. **Banapour B**, **Marthas ML**, **Munn RJ**, **Luciw PA**. 1991. In vitro macrophage tropism of pathogenic and nonpathogenic molecular clones of simian immunodeficiency virus (SIVmac). Virology **183**:12–19.

103. **Luciw PA**, **Shaw KE**, **Unger RE**, **Planelles V**, **Stout MW**, **Lackner JE**, **Pratt-Lowe E**, **Leung NJ**, **Banapour B**, **Marthas ML**. 1992. Genetic and biological comparisons of pathogenic and nonpathogenic molecular clones of simian immunodeficiency virus (SIVmac). AIDS Res Hum Retroviruses **8**:395–402.

104. **Mankowski JL**, **Flaherty MT**, **Spelman JP**, **Hauer DA**, **Didier PJ**, **Amedee AM**, **Murphey-Corb M**, **Kirstein LM**, **Muñoz A**, **Clements JE**, **Zink MC**. 1997. Pathogenesis of simian immunodeficiency virus encephalitis: viral determinants of neurovirulence. J Virol **71**:6055–60.

105. **Mori K**, **Rosenzweig M**, **Desrosiers RC**. 2000. Mechanisms for adaptation of simian immunodeficiency virus to replication in alveolar macrophages. J Virol **74**:10852–9.

106. **Flaherty MT**, **Hauer DA**, **Mankowski JL**, **Zink MC**, **Clements JE**. 1997. Molecular and biological characterization of a neurovirulent molecular clone of simian immunodeficiency virus. J Virol **71**:5790–8.

107. **Puffer BA**, **Pöhlmann S**, **Edinger AL**, **Carlin D**, **Sanchez MD**, **Reitter J**, **Watry DD**, **Fox HS**, **Desrosiers RC**, **Doms RW**. 2002. CD4 Independence of Simian Immunodeficiency Virus Envs Is Associated with Macrophage Tropism , Neutralization Sensitivity , and Attenuated Pathogenicity. J Virol **76**:2595–2605.

108. **Mori K**, **Ringler DJ**, **Kodama T**, **Desrosiers RC**. 1992. Complex determinants of macrophage tropism in env of simian immunodeficiency virus. J Virol **66**:2067–75.

109. **Borda JT**, **Alvarez X**, **Kondova I**, **Aye P**, **Simon MA**, **Desrosiers RC**, **Lackner AA**. 2004. Cell tropism of simian immunodeficiency virus in culture is not predictive of in vivo tropism or pathogenesis. Am J Pathol **165**:2111–2122.

110. **Sharma DP**, **Anderson M**, **Zink MC**, **Adams RJ**, **Donnenberg AD**, **Clements JE**, **Narayan O**. 1992. Pathogenesis of acute infection in rhesus macaques with a lymphocyte-tropic strain of simian immunodeficiency virus. J Infect Dis **166**:738–746.

111. **Clements JE**, **Mankowski JL**, **Gama L**, **Zink MC**. 2008. The accelerated simian immunodeficiency virus macaque model of human immunodeficiency virus-associated neurological disease: from mechanism to treatment. J Neurovirol **14**:309–317.

112. **Ortiz AM**, **Klatt NR**, **Li B**, **Yi Y**, **Tabb B**, **Hao XP**, **Sternberg L**, **Lawson B**, **Carnathan PM**, **Cramer EM**, **Engram JC**, **Little DM**, **Ryzhova E**, **Gonzalez-scarano F**, **Paiardini M**, **Ansari AA**, **Ratcliffe S**, **Else JG**, **Brenchley JM**, **Collman RG**, **Estes JD**, **Derdeyn CA**, **Silvestri G**. 2011. Depletion of CD4 + T cells abrogates post-peak decline of viremia in SIV-infected rhesus macaques. J Clin Invest **121**:4433–45.

113. **Micci L**, **Alvarez X**, **Iriele RI**, **Ortiz AM**, **Ryan ES**, **McGary CS**, **Deleage C**, **McAtee BB**, **He T**, **Apetrei C**, **Easley K**, **Pahwa S**, **Collman RG**, **Derdeyn C a.**, **Davenport MP**, **Estes JD**, **Silvestri G**, **Lackner AA**, **Paiardini M**. 2014. CD4 Depletion in SIV-Infected Macaques Results in Macrophage and Microglia Infection with Rapid Turnover of Infected Cells. PLoS Pathog **10**(10): e1004467. doi:10.1371/journal.ppat.1004467.

114. **Yen P-J**, **Mefford ME**, **Hoxie JA**, **Williams KC**, **Desrosiers RC**, **Gabuzda D**. 2014. Identification and characterization of a macrophage-tropic SIV envelope glycoprotein variant in blood from early infection in SIVmac251-infected macaques. Virology **458-459**:53–68.

115. **Yen P-J**, **Herschhorn A**, **Haim H**, **Salas I**, **Gu C**, **Sodroski J**, **Gabuzda D**. 2014. Loss of a conserved N-linked glycosylation site in the simian immunodeficiency virus envelope glycoprotein V2 region enhances macrophage tropism by increasing CD4-independent cell-to-cell transmission. J Virol **88**:5014–28.

116. **Carr JM**, **Hocking H**, **Li P**, **Burrell CJ**. 1999. Rapid and efficient cell-to-cell transmission of human immunodeficiency virus infection from monocyte-derived macrophages to peripheral blood lymphocytes. Virology **265**:319–29.

117. **Chen P**, **Hubner W**, **Spinelli MA**, **Chen BK**. 2007. Predominant Mode of Human Immunodeficiency Virus Transfer between T Cells Is Mediated by Sustained Env-Dependent Neutralization-Resistant Virological Synapses. J Virol **81**:12582–12595.

118. **Abela IA**, **Berlinger L**, **Schanz M**, **Reynell L**, **Günthard HF**, **Rusert P**, **Trkola A**. 2012. Cell-cell transmission enables HIV-1 to evade inhibition by potent CD4bs directed antibodies. PLoS Pathog **8**:e1002634.

119. **Kodama T**, **Wooley DP**, **Naidu YM**, **Kestler HW**, **Daniel MD**, **Li Y**, **Desrosiers RC**. 1989. Significance of Premature Stop Codons in env of Simian Immunodeficiency Virus. J Virol **63**:4709–4714.

CHAPTER 2

Derivation and Characterization of a CD4-Independent, Non-CD4 Tropic Simian Immunodeficiency Virus

Abstract

CD4 tropism is conserved among all primate lentiviruses and likely contributes to viral pathogenesis by targeting cells that are critical for adaptive anti-viral immune responses. Although CD4-independent variants of HIV and SIV have been described that can utilize coreceptors CCR5 or CXCR4 in the absence of CD4, these viruses typically retain their CD4 binding sites and can still interact with CD4. We describe the derivation of a novel CD4-independent variant of pathogenic SIVmac239, termed iMac239 that was used to create an R5-tropic SIV lacking a CD4 binding site. iMac239 contained 4 mutations in gp120 and 2 in the gp41 ectodomain. A single change (D178G) in the V1/V2 region was sufficient to confer CD4-independence, although additional mutations were required to stabilize this virus for a spreading infection. Like other CD4-independent viruses, iMac239 was highly neutralization sensitive, although mutations were identified that could confer CD4-independent infection without increasing its neutralization sensitivity. Strikingly, iMac239 retained the ability to replicate in cell lines and primary cells even when its CD4 binding site had been ablated by deletion of a highly conserved aspartic acid at position 385 that for HIV-1 plays a critical role in CD4 binding. iMac239, with and without the D385 deletion, exhibited an expanded host range in primary rhesus peripheral blood mononuclear cells that included $CCR5^+$, $CD8^+$ T cells. As the first non-CD4 tropic SIV, iMac239 will afford the opportunity to directly assess the in vivo role of CD4 targeting on pathogenesis and host immune responses.

Introduction

The primate lentiviruses, HIV-1, HIV-2 and SIV share a common mechanism of entry on target cells by interacting with CD4 and a member of the chemokine receptor family (1–3). CD4 binding to the envelope glycoprotein (Env) trimer initiates a cascade of conformational changes resulting in formation and exposure of the coreceptor binding site on gp120. Following coreceptor binding, gp41 is released to interact with the target cell membrane, leading to formation of a fusion intermediate and, ultimately, the 6-helix bundle, providing energy for membrane fusion and viral entry (1, 3–7). While CCR5, CXCR4, and, less commonly, other coreceptors can be used by these viruses during entry, CD4 tropism, mediated by a highly conserved binding site on gp120, is an invariant feature (1, 8), indicating that it likely plays a major role in pathogenesis. CD4 binding enables HIV-1 to evade host neutralizing antibody responses by limiting antibody access to neutralizing epitopes once the virion has attached to CD4 on the cell surface (9, 10). In addition, CD4 tropism in vivo focuses viral infection onto $CD4^+$ T cell subsets that are critical in mediating adaptive anti-viral immunity (11–14). These cells include Th1, Th17, T follicular helper, and T regulatory cells that collectively contribute to the coordinated induction, maturation and maintenance of cellular and humoral immune responses (15–24) and (for Th17 cells) to the integrity of the epithelial barrier at mucosal surfaces (18, 25, 26).

Although CD4-tropism is conserved, rare examples of CD4-independent viruses have been described that can utilize coreceptors, either CCR5 or CXCR4, for entry in the absence of CD4. These viruses, through mutations in gp120 and/or gp41, pre-form and expose a functional coreceptor binding site that is typically present only after CD4

binding occurs (27–38). By cryo-electron microscopy, Env trimers on CD4-independent viruses exhibit more open conformations compared to CD4-dependent viruses and in the absence of CD4 acquire conformations typically seen only after CD4 binding and triggering occur (39, 40). Although CD4-independent viruses have been derived in vitro (27–35, 41), they have only rarely been observed in vivo, as in rhesus macaques during late stage disease or following depletion of $CD4^+$ T cells prior to infection with anti-CD4 antibodies (42–44). CD4-independent viruses are typically highly neutralization sensitive, as a result of their more open Env trimer conformations and exposure of neutralization epitopes on cell-free virions that are poorly accessible after binding to the cell surface (10, 30, 37, 38, 45). Thus, CD4-independent viruses are likely strongly selected against in vivo (30, 46, 47). Nonetheless, although not strictly CD4-independent, HIVs and SIVs with the ability to utilize low levels of CD4 for entry are well-described, and this phenotype has been proposed to contribute to infection of macrophages in the periphery and microglial cells in the brain, which express lower levels of CD4 than T cells (30, 41–43, 48–55). For one neuropathic SIV isolate, its ability to cause AIDS encephalopathy in macaques correlated with infection of brain-derived endothelial cells that expressed CCR5 but not CD4 (56). Of note, viruses that are CD4-independent typically retain their CD4 binding site and the ability to engage CD4, if present (28, 32, 56–58), and have been shown to exhibit faster fusion kinetics in the presence of CD4 than CD4-dependent viruses (59).

For HIV-1, the CD4 binding site has been resolved at the atomic level and shown to be a deeply recessed pocket on gp120 formed by regions within the inner and outer domains that interact cooperatively with CD4 and gp41 during CD4 binding (1, 4, 39, 60,

61). Among HIV-1 and SIV isolates, some variability exists in these interactions. For SIVmac, the bulky side chain at Trp-375 has been shown to fill a space in the CD4 binding pocket reducing its dependency on CD4 binding, while HIV-1 contains a serine at this position that requires additional contributions from a layer on the gp120 inner domain (60). In HIV-1, residues Asp-368, Glu-370, and Tryp-427 are highly conserved and make multiple contacts with CD4, particularly amino acids Phe-43 and Arg-59 in its outermost D1 domain. Among these, Arg-59 forms a salt bridge with Asp-368 on gp120, and mutations in gp120 (8, 62) or CD4 (63, 64) that disrupt this bond ablate CD4 binding. Although crystallographic resolution of an SIV gp120 has not been determined, an aspartic acid at the analogous position (i.e. amino acid 385 for SIVmac) is conserved in all SIVs except for two SIV mandrill Env clones that contain a glutamic acid residue, suggesting that this aspartic acid is also critical for SIV gp120 interactions with CD4 (8) (Suppl. Fig. 2-1). In fact, a brain-derived isolate from a SIVmac239-infected macaque with an asparagine at this position, exhibited a 40-fold reduction in CD4 binding and CD4-independent use of CCR5 in a cell-cell fusion assay (65).

We describe the in vitro derivation of a novel variant of SIVmac239 that is both CD4-independent and unable to interact with CD4. This variant was adapted to replicate in a CD4-negative clone of SupT1 cells that expressed rhesus CCR5. Four mutations in gp120 and 2 in the gp41 ectodomain were associated with CD4-independence, and of these, a D178G mutation in the region analogous to the HIV-1 V1/V2 region was shown to be necessary for this phenotype, while additional mutations were required to stabilize the virus for a spreading infection. Although iMac239, like other CD4-independent viruses, was highly neutralization sensitive, mutations in gp120 were identified that

conferred CD4-independence in the absence of increased neutralization sensitivity. Notably, when Asp-385 was deleted to disrupt the CD4 binding site, fusion and infectivity of parental SIVmac239 were ablated while iMac239 remained fully replication competent on $CD4^+$, $CCR5^+$ cell lines and primary macaque lymphocytes. In addition, iMac239, with and without the D385 deletion, exhibited an expanded host range in primary macaque peripheral mononuclear cells that included $CCR5^+$, $CD8^+$ T cells. Thus, iMac239 will provide a novel platform for exploring the molecular and structural determinants for CD4-independence and neutralization sensitivity, and has enabled a novel non-CD4 tropic SIV variant to be derived that can be used to directly explore the role of CD4 binding and tropism in pathogenesis and in modulating host immune responses.

Methods

Cell Lines

Human SupT1 and BC7 cell lines (27) were transfected with a lentiviral vector to express rhesus CCR5 (RhCCR5)(66). SupT1/RhCCR5, BC7/RhCCR5, CEMx174, and HUT-78 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 2 mM penicillin-streptomycin (RPMI-Complete). The Japanese quail fibrosarcoma cell line QT6, the human embryonic kidney cell line 293T, and the human HeLa cell line TZM-bl engineered to express CD4 and CCR5 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from John C. Kappes) were cultured in Dulbecco's modified

Eagle medium supplemented with 10% FBS, 2 mM glutamine, and 2 mM penicillin-streptomycin (DMEM-Complete).

Env Cloning and Mutagenesis

Adapted *env* clones from SIV-infected BC7/RhCCR5 cultures were isolated as described previously (67). Mutant *env* genes were created using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's protocol. To repair a premature stop codon in the cytoplasmic tail of Env at position 734 this codon was reverted to the wild-type Q by QuikChange. To generate recombinant molecular clones of the SIVmac239 genome containing adapted and mutant *env* genes, *env* clones were cloned into the previously described pHVP-2 (also known as p239SpSp3') construct containing an open nef reading frame with a corrected HindIII site at position 602, and which contains the 3' half of the viral genome, through HindIII/SacI digest (68). Full-length genome constructs were then generated by cloning pVP-2 with p239SpSp5' as previously described (67) (p239SpSp5' was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Ronald Desrosiers). The identities of the recombinant clones were confirmed using restriction analysis and DNA sequencing. For the generation of luciferase reporter viruses, SIV *env* genes with a premature stop codon in the cytoplasmic tail (CT) coding region (Q734Stop) in pCR2.1 were digested with KpnI and XbaI and cloned into the similarly digested pCDNA3.1(-) expression construct.

Fusion Assays

Env fusion was assessed quantitatively on quail QT6 cells using a cell-cell fusion assay, expression constructs for CD4 and various coreceptors, and a reporter plasmid encoding luciferase under the control of a T7 promoter as has been previously described (67, 69, 70). Rhesus CD4 and coreceptors CCR5, CXCR6, APJ, GPR1, GPR15, CCR2 and CCR8 were kindly provided by Drs. Ronald Collman and Robert Doms.

Luciferase Reporter Viruses

Luciferase reporter viruses were generated, as previously described (71), by cotransfecting 293T cells with 8 μg of plasmid encoding the SG3 Δenv-based luciferase virus backbone and 4 μg of the appropriate env expression vector for 3-8hrs with the FuGENE6 transfection agent (Promega). Cell supernatants were collected at 48hrs post-transfection and stored at -80°C.

Virus Production

To generate molecularly cloned viruses, 293T cells were transfected with full-length viral genome constructs for 5hrs using calcium phosphate. Cell supernatants were collected 48 or 72hrs post-transfection and stored at -80°C. The uncloned iMac239 swarm was generated from supernatants of acutely infected BC7RhR5 cells, and stored at -80°C.

Viral Replication Assays

Virus concentrations were determined by enzyme-linked immunosorbent assay for viral p27 Gag antigen (Advanced Bioscience Laboratories). SupT1/RhCCR5 and BC7/RhCCR5 were inoculated with equivalent amounts of p27-containing virus. Following overnight incubation at 37°C, infected cells were washed in RPMI supplemented with 5% FBS to remove excess virus, and then maintained in RPMI Complete media. Viral replication was monitored by viral reverse transcriptase (RT) activity or quantity of p27 Gag in the culture supernatants.

Neutralization Assays

The sensitivity of luciferase reporter viruses bearing Envs of interest to neutralization by sera or plasmas from SIVmac251-infected rhesus macaques, soluble CD4, or monoclonal antibodies to SIVmac Envs, including murine antibodies 7D3, 8C7, 11F2, 17A11, 5B11, 4E11, 171C2, and 36D5 (72) or rhesus antibodies 1.4H, 6.10F, 6.10B, 9.1A, 1.7A, 1.10A, 2.6C, 3.11H (73–76), and 4.10F (unpublished, produced as in (75)) (kindly provided by Dr. James Robinson) was assessed in a TZM-bl pseudotype assay as previously described (71). Briefly, luciferase reporter viruses were incubated for 1hr at 37°C with various dilutions of serum, plasma, sCD4, or monoclonal antibodies and then used to infect TZM-bl cells pretreated with DEAE-dextran. Cells were incubated at 37°C for 48hrs and then lysed with the BriteLite Plus luminescence reporter assay system (Perkin Elmer). Infection was quantified by measuring luciferase activity with a Thermo LabSystems Luminoskan Ascent luminometer. Neutralization was measured as the reduction in luciferase activity compared with that of untreated controls.

Infection of PBMCs

Purified peripheral blood mononuclear cells (PBMCs) from rhesus macaques stored at -140°C were thawed and stimulated for 3 days with 5 μg/mL ConA at a concentration of 106 cells/mL in RPMI Complete media. Cells (5 x 106) were then inoculated with viruses (125 ng of p27 Gag) and media supplemented with IL-2 (100 IU/mL). After 24hrs, cells were washed to remove the viral inoculum and cultured in fresh RPMI Complete media supplemented with IL-2 (100 IU/mL).

Antibody Reagents

Antibodies used for surface staining included anti-CD14 BV650, anti-CD20 BV605, anti-CD8 BV570 (Biolegend), anti-CD16 APC Cy7, anti-CD95 PECy5, anti-CCR5 PE (BD Biosciences), anti-CD4 PECy5.5 (Invitrogen Life Technologies), and anti-CD28 ECD (Beckman Coulter). Antibodies used for intracellular staining included anti-CD3 V450 (BD Biosciences) and anti-p27 FITC (kindly provided by E. Rakasz, WNPRC).

Flow Cytometry Staining Assay

At peaks of viral replication, infected rhesus PBMCs were identified by p27 Gag positivity and flow cytometry. Aliquots of cells (1 x 106 per sample) were washed once with PBS and stained for viability with Aqua amine-reactive dye (Invitrogen) for 10 min in the dark at room temperature. A mixture of surface marker antibodies was added and kept at room temperature for 30 min in the dark. Cells were then washed with PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide and permeabilized

for 17 min at room temperature using the Cytofix/Cytoperm Kit (BD Biosciences). Immediately following permeabilization, cells were washed in Perm/Wash buffer (BD Biosciences) and a cocktail of antibodies for intracellular markers added and incubated in the dark for 1 hr at room temperature. Cells were then washed with Perm/Wash buffer, fixed with PBS containing 2% paraformaldehyde, and stored at 4°C until flow cytometric analysis. For flow cytometric analysis 3 x 105 events were acquired on an LSRII flow cytometer (BD Immunocytometry Systems) modified to detect up to 18 fluorophores. Antibody capture beads (BD Biosciences) were used to prepare compensation tubes for each individual antibody used in the experiment. Data analysis was performed using FlowJo Software version 9.0.1 (TreeStar).

Nucleotide Sequence Accession Number

The iMac239 *env* sequence has been deposited in GenBank under accession number KT959233.

RESULTS

Adaptation of SIVmac239 to CD4-negative BC7/RhR5 cells.

We derived a CD4-independent strain of SIV by serially passaging SIVmac239 on a 1:10 mixture of CD4-positive SupT1 cells and a CD4-negative variant of this line, BC7 (58) each of which stably expressed rhesus CCR5 (designated SupT1/RhR5 and BC7/RhR5, respectively). After 13 passages the virus could infect a pure culture of BC7/RhR5 cells and was then passaged an additional 8 times in this cell line. This viral swarm was able to replicate with high efficiency in both SupT1/RhR5 and BC7/RhR5,

while parental SIVmac239 could only replicate in the $CD4^+$ SupT1/RhR5 cells (Figure 2-1A). Env clones PCR-amplified from genomic DNA were derived, and their ability to mediate CD4-independent fusion using rhesus CCR5 evaluated on quail QT6 cells (69) (Fig. 2-1B). One clone (p8cl18) mediated comparable levels of fusion in the presence or absence of rhesus CD4 and was selected for further characterization. When inserted into a SIVmac239 backbone, this virus (iMac239 p8cl18 in Fig. 2-1C) was able to replicate with rapid growth kinetics in both SupT1/RhR5 (data not shown) and BC7/RhR5 cells (Fig. 2-1C), while parental SIVmac239 was only able to replicate in SupT1/RhR5 (Fig. 2-1A). CD4-independent replication in BC7/RhR5 cells also occurred when the expected premature stop codon acquired during passaging of this SIVmac in human cell lines (77) was corrected (Suppl. Fig. 2-2), and this Env was used to construct a virus containing a full length cytoplasmic tail, designated iMac239, that was employed in all subsequent experiments, except for production of pseudotypes, in which short CT Envs were used.

Mutations required for iMac239 CD4-independence

Sequencing of the iMac239 p18cl8 env revealed 7 coding changes from SIVmac239, 4 in gp120 and 3 in gp41 (Table 2-1, Suppl. Fig. 2-3). None of the gp120 mutations occurred in analogous regions of HIV-1 gp120 that contribute to the CD4 binding site, indicating that although the iMac239 Env and virus were CD4-independent, they likely retained the ability to interact with CD4. For gp120, three mutations (D178G, D337Y, and R427K) occurred within variable loop regions V1/V2, V3, and V4, respectively, while a single mutation (H224Q) occurred in a region analogous to the HIV-1 C2 domain flanking the V1/V2 stem. In gp41, two mutations in the ectodomain (K573T

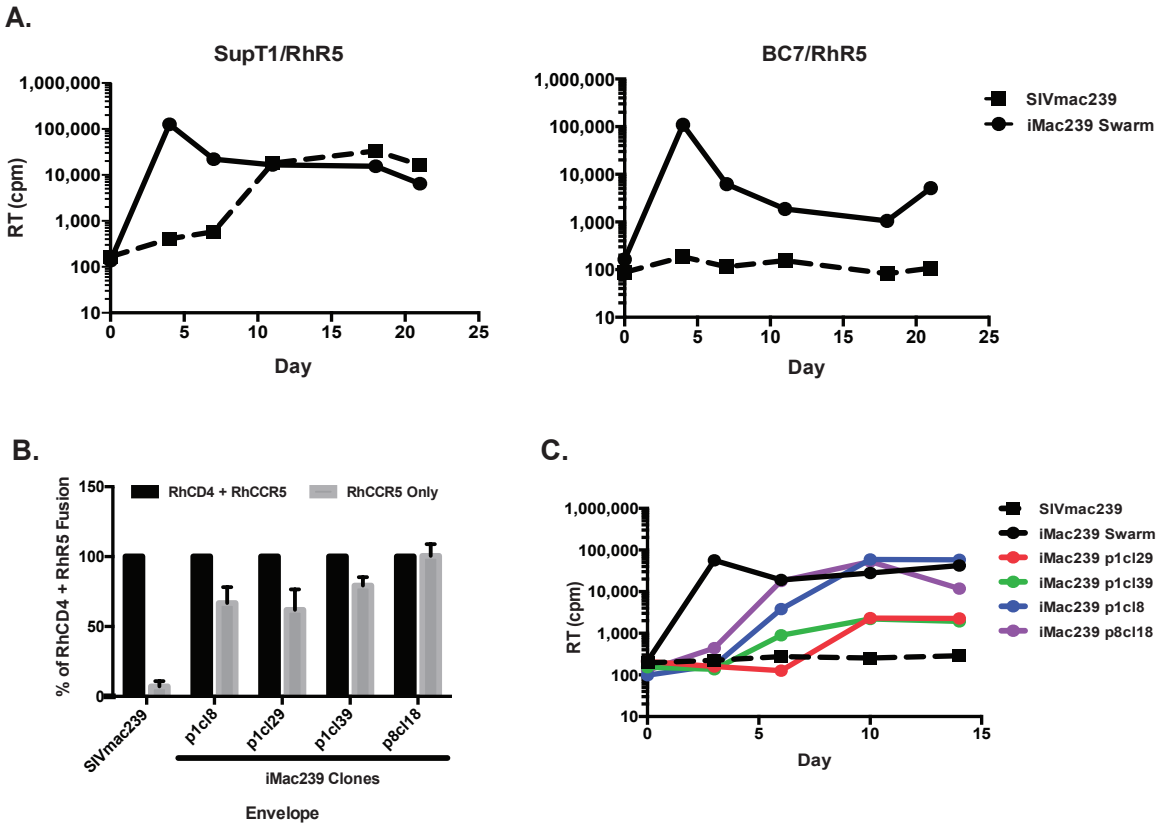

Figure 2-1: Replication and fusion of CD4-independent variants of SIVmac239. **(A)** Replication of parental SIVmac239 and an uncloned CD4-independent viral swarm is shown in CD4+, SupT1/RhR5 cells (Left panel) and CD4-negative BC7/RhR5 cells (Right panel) each of which stably expressed rhesus CCR5. RT (reverse transcriptase activity). **(B)** Fusion activity of SIVmac239 and four iMac239 Env clones is shown on QT6 cells using a cell-cell fusion assay. For each Env, the level of CD4-independent fusion on rhesus CCR5 is shown as a percentage of fusion (luciferase activity) in the presence of rhesus CD4. Background fusion levels on cells expressing only GFP were subtracted. The data shown are the means of three experiments plus the standard errors of the means (S.E.M.). **(C)** Growth curves in CD4-negative, BC7/RhR5 cells are shown for wildtype (WT) SIVmac239, the iMac239 viral swarm, and four recombinant SIVmac239-based viruses bearing the indicated iMac239 Env clones. RT activity in culture supernatants was measured at the indicated time points. Results from a representative experiment are shown.

Table 2-1: Amino acid differences in envelope glycoproteins between SIVmac239 and CD4-independent iMac239.

Region of Env	gp120				gp41		
	V1/V2	C2	V3	V4/V5	HR1	HR2	CT
A.A. Position	*178*	*224*	*337*	*427*	*573*	*673*	*820*
SIVmac239	D	H	D	R	K	N	L
iMac239	G	Q	Y	K	T	I	M

and N673I) were located within regions comparable to HIV-1 heptad-repeat domains 1 and 2 (HR1 and HR2), respectively, while one (L820M) occurred in the cytoplasmic tail.

The contributions of these mutations to CD4-independence were first evaluated in a cell-cell fusion assay (Fig. 2-2A). In the absence of CD4 the iMac239 Env generated fusion levels on CCR5 that were comparable to or slightly greater than in the presence of CD4, while parental SIVmac239 exhibited <10% fusion. When iMac239 mutations were introduced singly into the SIVmac239 Env, D178G in the V1/V2 loop was sufficient to confer CD4-independent fusion at levels approximately 50% greater than in the presence of CD4, although gp41 mutations K573T and N673I each produced modest increases in fusion to levels 40-50% of fusion in the presence of CD4.

We next evaluated CD4-independence in an infection assay on SupT1/RhR5 and BC7/RhR5 cells using viruses containing Envs with varying combinations of iMac239 mutations (Fig. 2-2B). When all 4 gp120 mutations were introduced into the SIVmac239 Env (SIVmac239 D178G H224Q D337Y R427K in Figure 2B), robust CD4-independent replication was observed in BC7/RhR5 cells with kinetics and levels that were comparable to a virus with the full iMac239 Env. However, a virus containing only the gp41 K573T and N673I mutations replicated poorly in both cell types (not shown). Interestingly, although the D178G mutation alone was sufficient to confer CD4-independence in the cell-cell fusion assay, a virus containing only this mutation replicated poorly in BC7/RhR5 cells and was noninfectious on $CD4^+$ SupT1/RhR5 cells (Fig. 2-2B). Virions from this virus exhibited similar levels of Env compared to both SIVmac239 WT and iMac239 virus, as measured by western blot, indicating that this defect was not the result of a failure of Env incorporation into virions (data not shown).

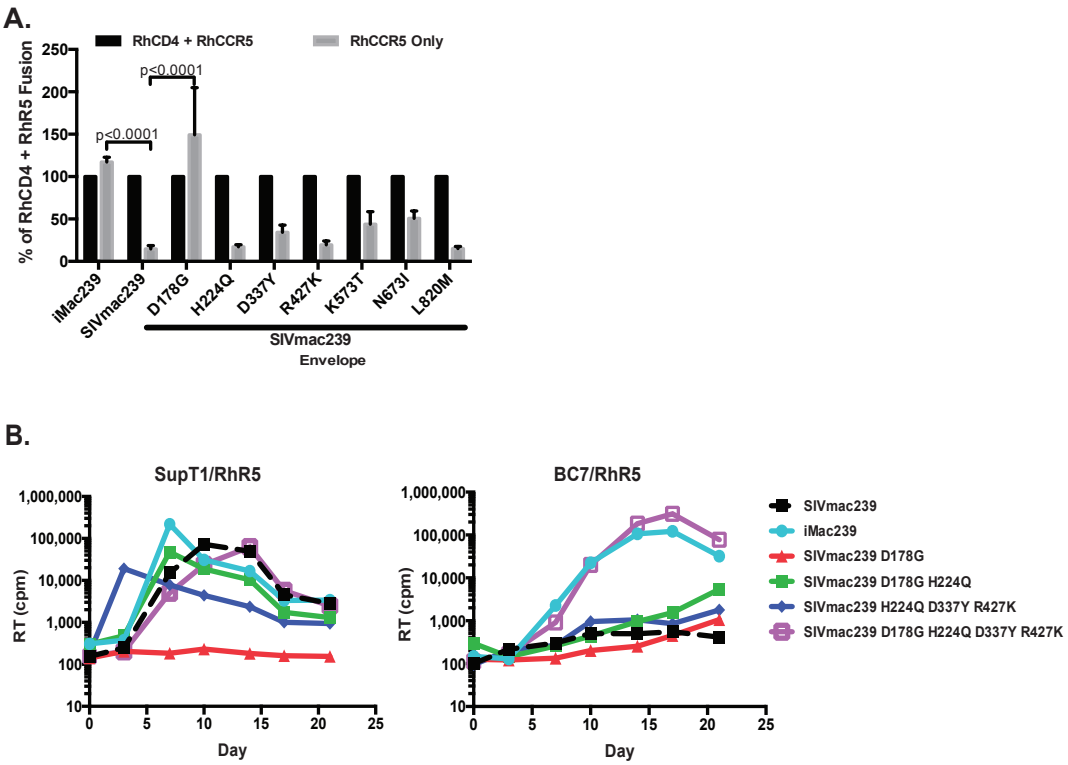

Figure 2-2: Determinants for iMac239 Env CD4-independence in cell-cell fusion and viral replication assays. (A) Fusion activity on rhesus CCR5 in the presence or absence of rhesus CD4 is shown for SIVmac239 Envs containing the indicated single mutations from iMac239. Data from 3 experiments + S.E.M are shown as in Fig. 1B. **(B)** Replication of SIVmac239-based viruses bearing the indicated Envs is shown in CD4+ SupT1/RhR5 (Left panel) and CD4- BC7/RhR5 cells (Right panel). Four changes in gp120 are sufficient to confer CD4-independent replication. A virus with D178G alone was unable to replicate in either cell type, but rescued for replication in SupT1/RhR5 by H224Q. RT activity was measured at the indicated time points. Results from a representative experiment are shown.

However, when viruses contained the D178G in combination with the gp120 H224Q mutation, replication was restored in both SupT1/RhR5 and BC7/RhR5 cells, although CD4-independent replication occurred more slowly in the latter (Fig. 2-2B). This apparent rescue of infectivity for virus containing the D178G alone was not seen when the other gp120 mutations were inserted individually (not shown). Significantly, removal of the D178G mutation from the iMac239 Env with all four gp120 changes resulted in a virus (SIVmac239 H224Q D337Y R427K) that was replication competent on SupT1/RhR5 cells but no longer CD4-independent and unable to infect BC7/RhR5 cells (Fig. 2-2B). Thus, among the gp120 mutations that conferred CD4 independence to SIVmac239, while D178G in V1/V2 was critical, this mutation alone resulted in a virus that was noninfectious in both CD4-positive and -negative cell types but could be rescued by the H224Q change in gp120.

Neutralization sensitivity of iMac239

For HIV-1 and SIV, CD4-independent Envs are typically highly neutralization sensitive, owing to their more open conformation of the Env trimer on virions (39, 40) and formation of highly immunogenic epitopes that are typically induced only in the presence of CD4 (9, 30, 37, 38). Given the well-described neutralization resistance of the SIVmac239 Env, we were interested in determining the sensitivity of the iMac239 Env to a panel of sera and plasmas from SIVmac-infected rhesus macaques and to a panel of anti-SIVmac gp120 murine monoclonal antibodies, previously shown to potently neutralize lab-adapted SIVmac251 but not SIVmac239 (72). Neutralization assays were performed on TZM-bl cells using viral particles pseudotyped with Envs. In addition to

iMac239, we also evaluated SIVmac239 Envs containing the 4 gp120 changes that were sufficient to confer CD4 independence (Fig. 2-2B) in viral replication assays, and an Env containing only the D178G, which was CD4-independent in the cell-cell fusion assay (Fig. 2-2A).

As expected, whereas SIVmac239 was resistant to neutralization by anti-SIV sera or plasma, with inhibitory dilutions (ID50) <30, iMac239 was highly sensitive with ID50s >2 million for plasma and 8,793 for sera (Fig. 2-3). Similar results were seen with the panel of monoclonal antibodies, with inhibitory concentrations (IC50) >10-26 μg/ml for SIVmac239 and <0.0003 μg/ml for the 3 antibodies tested. Surprisingly, iMac239 Envs containing the minimum number of mutations in gp120 that conferred CD4 independence in either cell-cell fusion or viral infection assays remained neutralization resistant at levels comparable to parental SIVmac239. These findings indicate that although typically associated, CD4-independence and increased neutralization sensitivity can be dissociated. Moreover, these findings also suggest that changes in the iMac239 gp41 that were selected for in vitro and not present in the SIVmac239 D178G H224Q D337Y R427K Env used in this assay were key determinants for its marked neutralization sensitivity.

CD4-independence of iMac239 is retained following ablation of the CD4 binding site

Although CD4-independent Envs are structurally altered and expose or form neutralization epitopes (30, 37–40, 45), as noted above, the iMac239 Env on virions likely retained a CD4 binding site. In order to determine if the iMac239 Env would remain competent for fusion and infection even after its CD4 binding site had been

		SIVmac239	iMac239	SIVmac239 D178G	SIVmac239 D178G H224Q D337Y R427K
Plasma/Serum	23696 Pool A	270	>2,343,750	358	442
	23166 PoolA	<30	>2,343,750	46	47
	24724 Pool B	<30	>2,343,750	58	69
	P309 Serum	<30	8,703	<30	<30
CD4-induced Epitope Antibodies	7D3	>26	<0.0003	9.34	11.82
	11F2	>10.33	<0.0001	>10.33	>10.33
	1.4H	>15.67	<0.0002	>15.67	>15.67

Plasma Dilutions (ID_{50})	<100	100 - 1,000	1,000 - 100,000	>100,000

IC_{50} Values (ug/mL)	>2	0.2 - 2	<0.01 - 0.2

Figure 2-3: Neutralization sensitivity of Envs with iMac239 mutations. Viral pseudotypes containing the indicated Envs were preincubated with varying dilutions of plasma, serum, or monoclonal antibodies prior to infection of TZM-bl cells. Inhibitory dilutions (ID_{50}s) for plasma and serum are color-coded (<100, green; 100-1,000, yellow; 1,000-100,000, orange; >100,000 red). Inhibitory concentrations of monoclonal antibodies (IC_{50}s) are color-coded (>2 ug/mL, green; 0.2-2 ug/mL, yellow; <0.01-0.2 ug/mL red).

ablated, we introduced a 3 nucleotide deletion removing a codon for an aspartic acid at amino acid position 385 that is highly conserved throughout HIV and SIV phylogeny (8) (Suppl. Fig. 2-1). For HIV-1, the analogous Asp at position 368 (HXB numbering) forms a salt bridge with arginine 59 of CD4 (1, 2), and a D368R mutation in HIV-1 gp120 ablates CD4 binding and most CD4 binding site epitopes (8, 62).

The effects of the D385 deletion (ΔD385) on SIVmac239 and iMac239 Envs were assessed in cell-cell fusion assays and on viral replication on CD4-positive and -negative cell lines. Remarkably, whereas the ΔD385 largely ablated fusion of SIVmac239 Env on target cells bearing CD4 and CCR5 to levels <10% of wildtype, iMac239 fusion was unaffected and was actually enhanced in the presence of this mutation (Fig. 2-4A). When viral replication was assessed in SupT1/RhR5 and BC7/RhR5 cells, SIVmac239 containing the ΔD385 mutation was unable to replicate in either cell type, whereas iMac239 with or without the ΔD385 mutation replicated in both cell types with similar kinetics (Fig. 2-4B Left and Right panels). We confirmed that viruses used in these infection assays expressed comparable amounts of gp120 relative to p27 Gag (not shown).

The sensitivity of viral pseudotypes bearing these Envs to neutralization by soluble CD4 (sCD4) was also assessed as an indicator of CD4 binding to Env trimers on virions. Infectivity of pseudotypes containing SIVmac239, SIVmac251.6 (a lab-adapted SIVmac), iMac239, or iMac239-ΔD385 Envs was evaluated on TZM-bl cells in the presence of varying concentrations of sCD4. While sCD4 sensitivity was observed for SIVmac239 (IC50, 7.8 μg/ml) and markedly enhanced for SIVmac251.6 (IC50, 0.1 μg/ml) and iMac239 (IC50 <0.01 μg/ml), iMac239 containing the ΔD385 mutation was highly resistant (IC50, >20 μg/ml) (Fig. 2-4C). Collectively, these findings indicate that

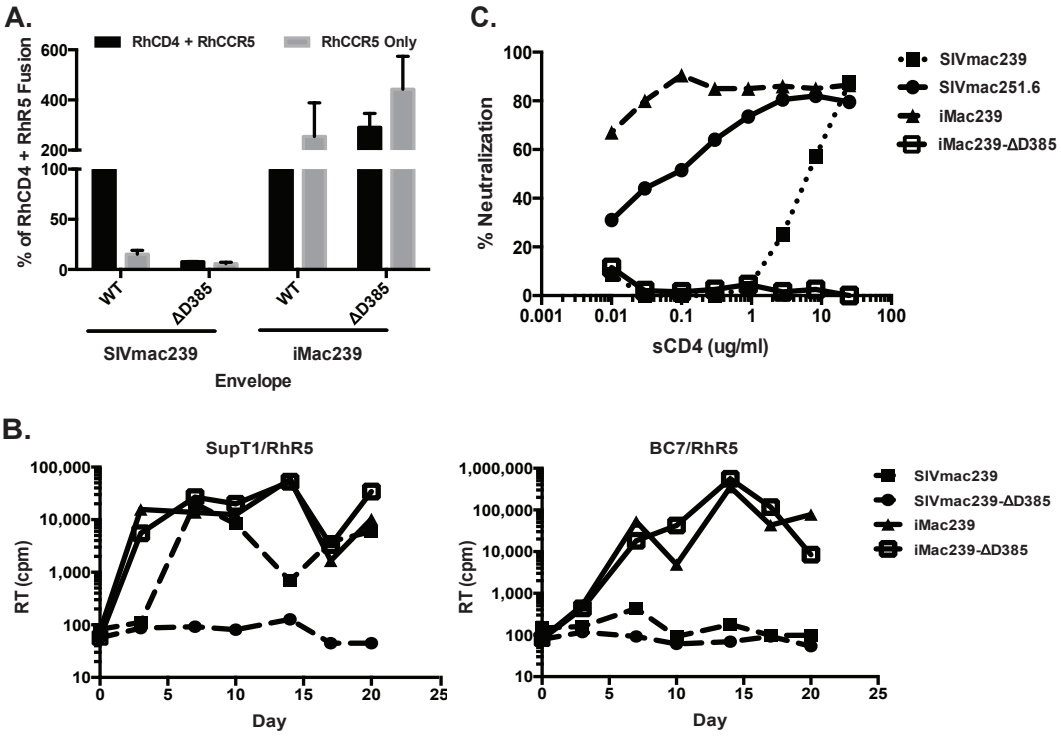

Figure 2-4: Effect of the ΔD385 mutation in cell-cell fusion, viral replication, and neutralization assays. (A) Fusion activities of SIVmac239, SIVmac239-ΔD385, iMac239, and iMac239-ΔD385 on rhesus CCR5 only are shown for each Env as the percentage of fusion in the presence of rhesus CD4. Background was subtracted as in Fig. 1B. The data shown are the means of four experiments + S.E.M. **(B)** Replication of SIVmac239, SIVmac239-ΔD385, iMac239, and iMac239-ΔD385 viruses in CD4+ SupT1/RhR5 cells (Left panel) and CD4- BC7/RhR5 cells (Right panel). RT activity in culture supernatants was measured at the indicated time points. Results from a representative experiment are shown. **(C)** Soluble CD4 (sCD4) neutralization of viral pseudotypes containing SIVmac239, SIVmac251.6, iMac239, and iMac239-ΔD385 Envs is shown on TZM-bl cells. Percent neutralization was calculated using luciferase activity normalized to infection in the absence of sCD4. Results from a representative experiment are shown.

although the iMac239 Env contained its CD4 binding site and was highly sensitive to sCD4 neutralization, this Env could mediate entry while lacking a CD4 binding site.

Identifying neutralization epitopes on iMac239 and iMac239-ΔD385.

Given the exquisite sensitivity of iMac239 Env to sera from SIVmac-infected macaques and monoclonal antibodies to CD4-induced epitopes and the ability of iMac239 virus to replicate without a functional CD4 binding site, we sought to determine if iMac239's neutralization sensitivity could be mapped to particular epitopes, and if conformational changes associated with its altered antigenicity were affected by the loss of the CD4 binding site. Neutralization of viral particles pseudotyped with Envs from SIVmac239, iMac239 or iMac239-ΔD385 was assessed on TZM-bl cells using a panel of monoclonal antibodies to SIVmac variable loops (V2, V3 and V4), and to the CD4 and CCR5 binding sites (72–76). As shown (Fig. 2-5), while SIVmac239 was largely resistant to all antibodies tested, iMac239 was sensitive to 10 of 13 antibodies and resistant only to an anti-CD4/CCR5 binding site (17A11), an anti-V2 (171C2), and an anti-V4 loop (1.7A) antibody. The iMac239-ΔD385 Env was also highly neutralization sensitive at levels that were comparable to or greater than iMac239. Thus, relative to SIVmac239, CD4-independent iMac239 was globally neutralization sensitive to multiple antibodies, and this sensitivity was further enhanced by the ΔD385 mutation. These findings also indicate that while the ΔD385 mutation largely ablated CD4 binding function, it did not disrupt the antigenicity of the CD4 binding site, as determined by the antibodies in our panel.

	SIVmac239	iMac239	iMac239-ΔD385	Epitope
6.10B	>34.5	0.1	0.08	CD4 Binding Site
5B11	34.66	0.35	0.08	CD4 Binding Site
17A11	28.43	6.61	0.29	CD4/CCR5 Binding Site
4E11	23.68	0.12	0.02	CD4/CCR5 Binding Site
7D3	30.6	0.0004	0.001	CCR5 Binding Site
171C2	19.28	22	0.8	V2 Loop
6.10F	>17.5	0.002	0.005	V3 Loop
36D5	6.93	0.013	0.02	V3 Loop
3.11H	>50	0.01	0.02	V3 Loop
1.7A	>26	20.85	5.84	V4 Loop
4.10F	>20.5	2	0.09	V4 Loop
1.10A	>50	0.0003	0.001	V4 Loop
9.1A	>22.5	0.001	0.001	V4 Loop

IC_{50} values (ug/mL)	>2	0.2 - 2	<0.01 - 0.2

Figure 2-5: Neutralization of viral pseudotypes by monoclonal antibodies. Neutralization of viral pseudotypes bearing SIVmac239, iMac, or iMac-ΔD385 Envs by the indicated monoclonal antibodies is shown. IC_{50}s are shown and color-coded (>2 ug/mL, green; 0.2-2 ug/mL, yellow; <0.01-0.2 ug/mL red).

Replication of iMac239 and iMac239-ΔD385 in primary rhesus macaque PBMC.

Given the ability of CD4-independent iMac239 to replicate in T cell lines with or without a CD4 binding site, we assessed the infectivity of iMac239 and iMac239-ΔD385 on primary rhesus PBMCs. Similar to T cell lines, parental SIVmac239 containing the ΔD385 mutation was completely noninfectious. However, both iMac239 and iMac239-ΔD385 replicated to levels identical to SIVmac239, albeit with a delay to peak of 3-6 days (Fig. 2-6A), indicating that a CD4 binding site was not required for this virus to infect primary cells.

Next, given the potential for CD4-independent iMac239 and iMac239-ΔD385 to have an expanded cellular tropism, we assessed their infectivity on mitogen (ConA) and IL-2 stimulated rhesus macaque PBMC using flow cytometry and a panel of antibodies to T, B and monocyte subsets and to CCR5. Peak intracellular viral p27 Gag expression occurred at different days post inoculation with SIVmac239 infection peaking at 4 dpi and iMac239 and iMac239-ΔD385 peaking at 10 dpi (Fig. 2-6A). Gating strategies are shown on uninfected cells (Suppl. Fig. 2-4). Among CD3+ T cells assayed at the viral peak, iMac239 infection produced a significant increase in p27 Gag-positive cells compared to both SIVmac239 and iMac239-ΔD385 (i.e., 25.4% vs. 5.56% and 6.46%, respectively, in the representative experiment shown in Fig. 2-6B). Among p27 Gag+, CD3+ T cells, the vast majority (>90%) of SIVmac239-infected cells were negative for CD4 and CD8, most likely reflecting $CD4^+$ T cells from which CD4 was downregulated by the effects of Nef and Env expression (78–81), and only rare cells (<1%) expressed CD8. In marked contrast, for iMac239 and iMac239-ΔD385 infections, on average 60% and 40% of p27 Gag+ cells, respectively, were CD8-positive (Fig 6C). Infection of

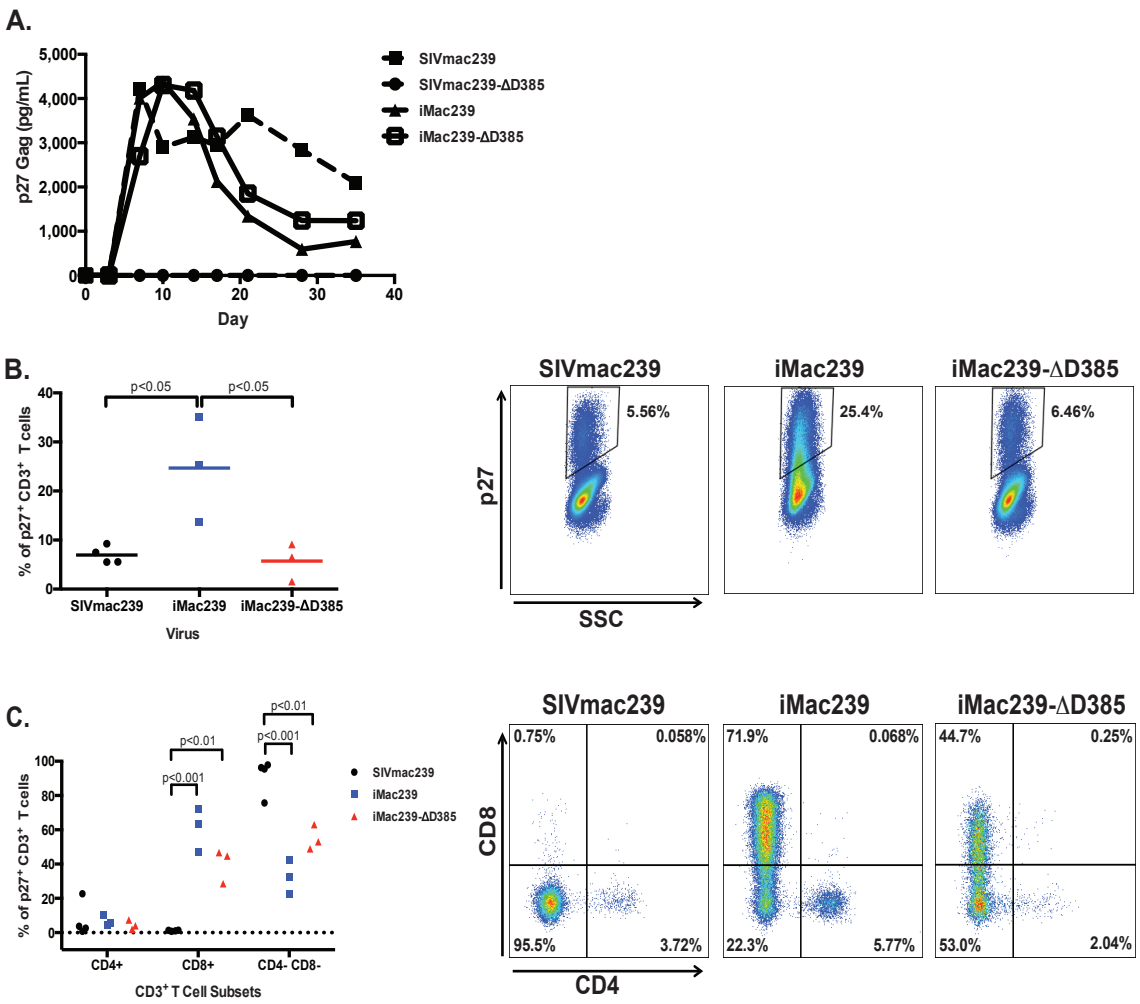

Figure 2-6: Replication of SIVmac239 and iMac239 with and without the ΔD385 mutation in rhesus PBMCs. (A) Replication of SIVmac239, SIVmac239-ΔD385, iMac239, and iMac239-ΔD385 in ConA/IL-2 stimulated rhesus PBMCs is shown. p27 Gag in culture supernatants was quantified by ELISA at the indicated time points. Results from a representative experiment are shown. **(B)** For each virus, the percentage of total CD3+ T cells that are positive for p27 Gag are indicated (Left panel). Flow cytometry cytograms from a representative experiment show the percentage of p27 Gag+, CD3+ T cells (Right panel). **(C)** The percentage of p27 Gag+, CD3+ T cells that express CD4 and/or CD8 at peak infection is shown (Left panel). Cytograms from a representative experiment show that for iMac239 and iMac239-ΔD385, a marked increase in p27 Gag is detectable in CD8+ T cells (Right panel).

monocytes (CD16+, CD14+) or B cells (CD20+) was not observed (data not shown), although we note that culture conditions did not support expansion of these cell types. Collectively, these data indicate that both CD4-independent iMac239 and iMac239-ΔD385 have expanded tropism on primary cells, specifically for $CD8^+$ T cells.

Evaluating use of alternative coreceptors by iMac239 and iMac239-ΔD385.

SIVmac239 and other SIVs have been well described to use coreceptors in addition to CCR5, including CXCR6, APJ, GPR1, GPR15, CCR2 and CCR8 (82–86). To determine if CD4-independent use of CCR5 by iMac239 and iMac239-ΔD385 affected CD4-dependent or independent use of alternative coreceptors, a cell-cell fusion assay was used to assess fusion on target cells expressing rhesus CXCR6, APJ, GPR1, GPR15, CCR2 and CCR8 with or without rhesus CD4. In the presence of CD4, SIVmac239, iMac239, and iMac239-ΔD385 exhibited some capacity to use CXCR6, GPR1 and GPR15, although levels of fusion were less than for CCR5 (Fig. 2-7A Left panel). However, in the absence of CD4, only iMac239 and iMac-ΔD385 exhibited CD4-independent fusion, and only on CCR5 (Fig. 2-7A Right panel).

We also assessed infection of two $CD4^+$, CCR5-negative cell lines, CEMx174 and HUT-78, previously shown to be permissive for SIVmac infection, most likely through their expression of GPR15 (86–91). In contrast to SIVmac239, both iMac239 and iMac239-ΔD385 were unable to replicate in these cell lines (Fig. 2-7B). Thus, while adapted for CD4-independent use of rhesus CCR5, these findings suggest that iMac239 and iMac239-ΔD385, are strictly CCR5 tropic and unable to use alternative coreceptors for infection in the presence or absence of CD4.

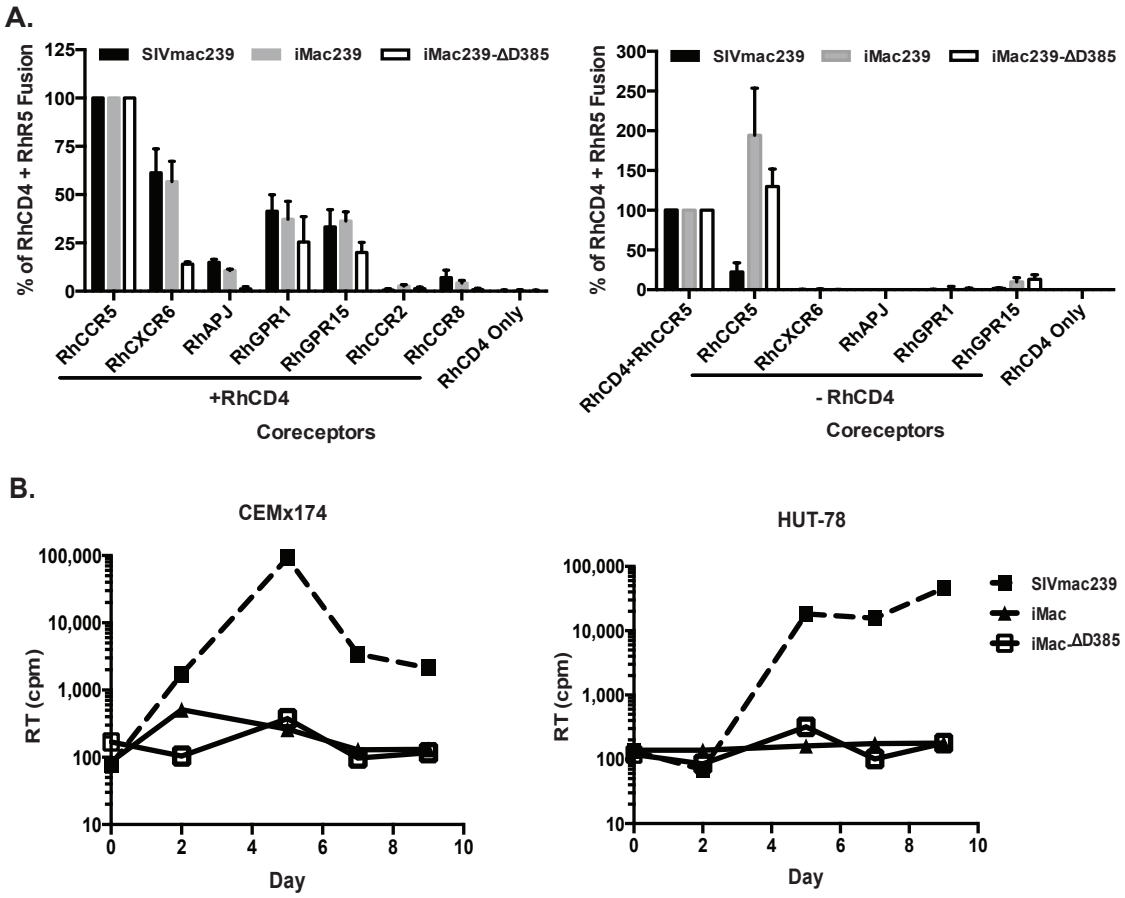

Figure 2-7: Use of alternative coreceptors by SIVmac239, iMac239, and iMac239-ΔD385. **(A)** Fusion activity of the indicated Envs on rhesus coreceptors in the presence (Left panel) or absence (Right panel) of rhesus CD4 was assessed in a cell-cell fusion assay. In each panel luciferase activity was normalized to values for rhesus CCR5. Background fusion levels were subtracted prior to normalization. Data shown are the means of three experiments + S.E.M. **(B)** Replication of SIVmac239, iMac239, and iMac239-ΔD385 viruses in CD4+, CCR5- CEMx174 (Left panel) and HUT-78 (Right panel) cells. RT activity in culture supernatants was measured at the indicated time points. Results from a representative experiment are shown.

DISCUSSION

CD4 tropism is conserved among all primate lentiviruses and has been proposed to play a key role in protecting viruses from neutralizing antibodies that are sterically restricted from accessing the Env trimer once virions have bound to the cell surface (9, 10). However, by focusing infection onto cells that are critical to host adaptive immune responses, CD4 tropism likely also exerts potent immunomodulatory effects that contribute to disease and/or viral persistence. Although CD4-independent viruses have been observed in vivo, particularly in nonhuman primate models of pathogenic SIV infection, they typically appear in the setting of highly immunocompromised hosts with advanced neurological or pulmonary complications (42, 56, 65) at sites where non-lymphoid cells with little or no CD4 are infected. In rhesus macaques depleted of CD4 T cells with anti-CD4 antibodies prior to SIVmac infection, CD4-independent viruses rapidly appeared, in association with encephalopathy and macrophage infection (44, 51), indicating that this phenotype can readily emerge in vivo. Because CD4-independent viruses are characteristically neutralization sensitive, it is likely that they are strongly selected against during typical pathogenic infection (30, 46, 47). Interestingly, primary isolates of HIV-2, which is less pathogenic than HIV-1 (reviewed in (92)), have been reported to exhibit CD4-independence in vitro (57, 93). However, for HIV-1 (and as we show for SIVmac239) extensive passaging is required to derive CD4-independent viruses in vitro, indicating that for these viruses there are likely to be additional barriers to their emergence (27, 28, 32, 34, 35, 37, 45). Of note, CD4-independent Envs typically retain their CD4 binding site, and their infectivity is generally enhanced in the presence of CD4 (28, 30, 44, 57). Thus, while CD4-independent viruses arising in vivo or in vitro provide

potentially useful tools to understand conformational changes associated with coreceptor engagement and viral entry (32, 33, 37, 38, 59), they have not been able to be applied to address questions of what role CD4 interactions play in pathogenesis and on host immune responses.

In this report we describe the derivation and characterization of a CD4-independent and truly non-CD4 tropic variant of SIVmac239 that lacks the ability to interact with CD4. A CD4-independent virus, iMac239, was first derived in vitro and shown to be highly competent in mediating fusion and infection of cells bearing rhesus CCR5 in the absence of CD4. Unlike SIVmac316, a macrophage tropic variant of SIVmac239 that was CD4-independent in cell-cell fusion assays (30, 42), the determinants for iMac239's altered tropism resided solely within gp120, and this virus did not require a truncated cytoplasmic tail to exhibit this phenotype. Notably, after deletion of the codon for a highly conserved aspartic acid in the CD4 binding loop on gp120, shown for HIV-1 to be critical for CD4 binding, iMac239 remained fully infectious on CD4-negative cell lines expressing rhesus CCR5 and on primary peripheral blood lymphocytes. This mutation in parental SIVmac239 completely ablated its function in cell-cell fusion and infection assays. Moreover, whereas SIVmac239 and especially iMac239 were sensitive to neutralization by soluble CD4, iMac239 containing the ΔD385 deletion was completely resistant, consistent with the view that CD4 binding for this virus was ablated or at least markedly reduced (Fig. 2-4).

Among the 7 mutations in the iMac239 Env, 4 changes in gp120 were sufficient to confer CD4-independent infection of CCR5-expressing cells. A D178G in the iMac239 V1/V2 loop was critical in that this change alone conferred CD4-independent fusion to

the SIVmac239 Env, and correction of this change alone ablated CD4-independent infection by a virus bearing the minimum set of gp120 mutations required for CD4-independence. For HIV-1 and SIV, changes in V1/V2 are frequently associated with CD4-independence (28, 31–33, 41, 94–96) and/or an enhanced ability to infect cells that express low levels of CD4 (93, 97). Structural studies of HIV-1 soluble SOSIP timers (98–100) and cryo-electron microscopic analyses of virion-associated trimers have shown, in the absence of CD4, V1/V2 loops to be oriented towards the apex of the trimer, in contrast to their more lateral positioning upon CD4 activation (39). Given that soluble gp120, even in the absence of CD4, is thermodynamically favored to assume a CD4 bound conformation as an apparent default structure (101) even though its conformation is likely restrained by the V1/V2 and V3 variable loops (99–101), changes in V1/V2 that perturb its quaternary interactions within or between adjacent protomers could favor the spontaneous opening of the trimer to a CD4 bound conformation and promote CD4-independent function. Interestingly, although D178G alone enabled the SIVmac239 Env to fuse independently of CD4, SIVmac239 virus containing only this change was noninfectious on both CD4-positive and -negative cells. However, this Env could be rescued by iMac239's H224Q mutation distal to the V1/V2 stem. Because the Env trimer has been modeled as a metastable structure with the potential to assume conformations that are either favorable or non-permissive for fusion (102, 103), we interpret these results to indicate that D178G, while necessary for CD4-independent fusion and entry, requires the H224Q to guide conformational changes towards a fusion-permissive rather than an inactive state, similar to that described for HIV-1 Envs after cold treatment (102) and/or small molecule CD4 binding site agonists (103–105).

As noted, it is likely that the enhanced neutralization sensitivity of CD4-independent viruses results from their more open structure because their Env trimers assume conformations that typically only occur in the presence of CD4, exposing epitopes that are shielded on resting virions (39). In addition, CD4-induced epitopes that contribute to the coreceptor binding site and are highly immunogenic (9), are poorly formed in the absence of CD4 binding and inaccessible to antibodies on cell-bound virions. However these epitopes are targeted on CD4-independent viruses on which they are formed and exposed (37) or sampled more frequently in the absence of CD4 (106). As we demonstrated, iMac239, as well as its non-CD4 binding derivative, iMac239-ΔD385, were globally neutralization sensitive to sera from SIVmac-infected animals and to monoclonal antibodies to CD4-induced and non-induced epitopes (Figs. 2-3 and 2-5). However, an Env containing only the iMac239 gp120 changes, while CD4-independent, remained highly neutralization resistant, similar to parental SIVmac239. In addition to indicating that CD4-independence and enhanced neutralization sensitivity can be dissociated, these findings also suggest that changes in gp41 that arose with iMac239's CD4-independence contribute to its neutralization sensitivity. This finding is consistent with the model of "intrinsic reactivity" of the Env trimer proposed by Haim, et al. (102), in which changes in gp41 enhanced the spontaneous formation/exposure of the HR1 coiled coil, decreasing the threshold for Env to transition upon activation from a high to a lower-energy state.

As described, CD4-independent iMac239 virus, following deletion of aspartic acid 385, remained fully infectious on CCR5-expressing cell lines and on primary lymphocytes. Although the structure of the SIVmac gp120 has not been resolved at the

crystallographic level, for HIV-1, this residue forms a covalent bond with arginine-59 on human CD4 (corresponding to lysine-59 on rhesus CD4), and is highly conserved across nearly all HIV and SIV isolates ((8) and see Suppl. Fig. 2-1). While we cannot rule out the possibility that iMac239 containing this mutation maintained some low level interactions with CD4, the finding that it became completely resistant to soluble CD4 while iMac239 was exquisitely sensitive, strongly supports the view that CD4 binding was markedly impaired (Fig. 2-4). We chose to introduce a deletion rather than a point mutation at this position to create a CD4-binding site mutant that would be less likely to revert in vivo in macaques. In vitro, when iMac239-ΔD385 was serially passaged up to 20 times in $CD4^+$ SupT1/RhR5 cells, this mutation remained stable (not shown) indicating that loss of CD4 binding function, at least in cell lines, did not confer a major fitness cost during long-term propagation in vitro.

In rhesus PBMCs cultured with T cell mitogens, SIVmac has been shown to infect CD4 effector and central memory T cells, consistent with expression of CCR5 on these cells and SIVmac's highly efficient use of this coreceptor for entry (47, 107, 108). Although alternative coreceptors can be used by SIVs in vivo (82, 109), it is likely that levels of CCR5 expression are a key determinant of tropism and pathogenicity, given that sooty mangabeys, a natural host for nonpathogenic SIVsm infection, exhibit low CCR5 expression on central memory CD4 T cells, likely accounting for sparing of this subset in the context of SIVsm infection (108, 110). Among peripheral blood cells stimulated with T cell mitogens, iMac239 with and without the ΔD385 deletion, exhibited an expanded host range that included CD8 T cells, most likely through their expression of CCR5 (Fig. 2-6). We observed that 30 - 65% of CD8 cells in these cultures expressed CCR5 (not

shown), which was associated with infection of approximately 20% and 4% of CD3+, $CD8^+$ T cells by iMac239 and non-CD4 tropic iMac239-ΔD385, respectively, in contrast to <0.15% for SIVmac239. Adaptation of SIVmac239 for CD4-independent use of CCR5 led to a reduced capacity to utilize alternative coreceptors (Fig. 2-7), suggesting that its expanded tropism in vitro was largely driven by CCR5 expression. Collectively, these findings clearly show that the tropism of SIVmac239 on primary cells can be altered and redirected from its exclusive infection of $CD4^+$ target cells. Whether additional cell types such as NK, B cells, or monocytes can be infected by this virus remain to be determined.

The ability to remove CD4 tropism from SIVmac creates new opportunities to assess the role of CD4 in pathogenesis. Non-human primate models of AIDS have clearly shown that during early SIV infection, $CD4^+$ T cells that express CCR5 and reside in mucosal tissues are selectively and rapidly depleted (107, 111, 112), which is associated with a disruption in the epithelial barrier that contributes to microbial translocation and systemic immune activation (18, 25, 26, 113, 114). In addition, by focusing infection onto T cell subsets that provide help for adaptive immune responses, including Th1, Th17, and Tfh cells, it is likely that CD4 tropism has profound effects on antiviral immune responses, which are ultimately inadequate to contain viral replication and disease progression. Binding of gp120 to CD4 also has the potential to disrupt CD4's physiologic interaction with HLA class-II on antigen presenting cells, which underlies T-cell immunologic helper functions. Although iMac239-ΔD385 exhibited expanded cell tropism in vitro, its inability to selectively target $CD4^+$ T cell subsets raises the possibility that T cell help for cytotoxic CD8 and CD4 cellular responses will be qualitatively or qualitatively altered and that B cell maturation and memory responses, which are

dependent on interactions with T follicular helper cells, may lead to improved antibody responses. Future studies that assess the quality of anti-SIV responses in the context of a CD4 sparing infection will provide new insights into pathogenesis and possibly inform interventions that can be directed to improve host immune responses to infection and vaccines.

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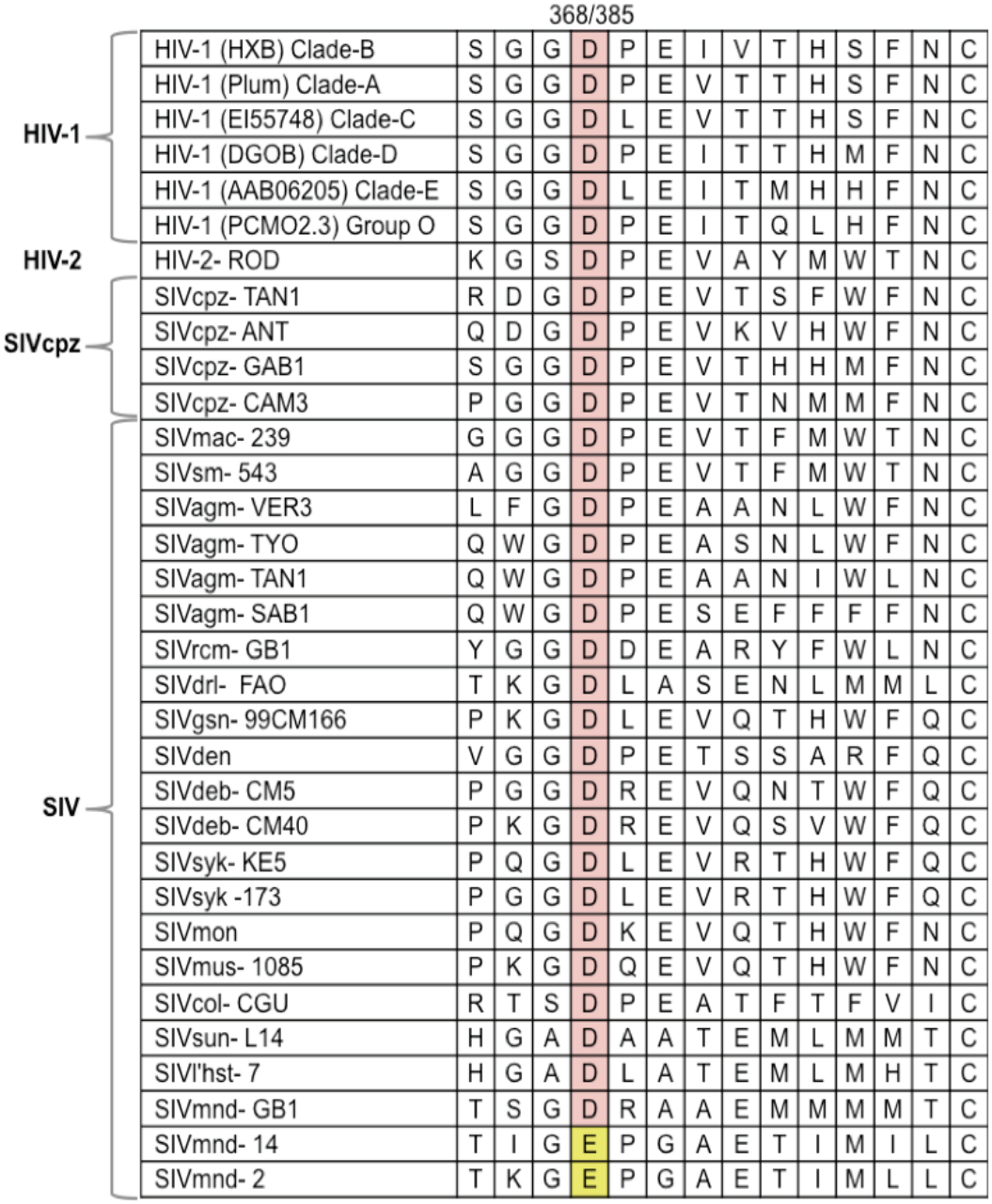
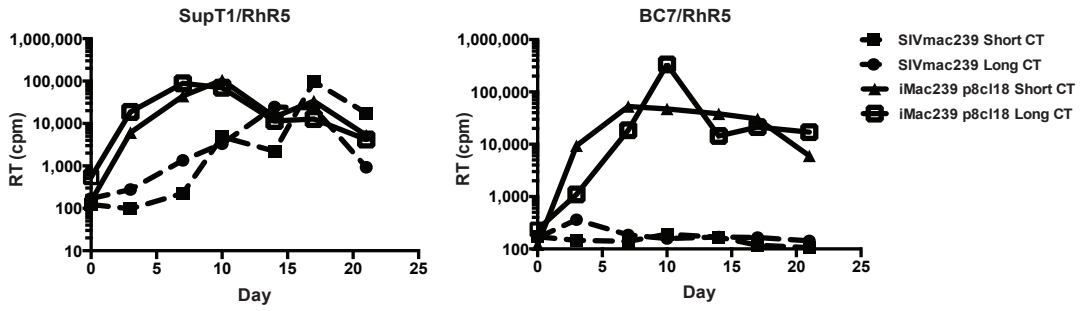
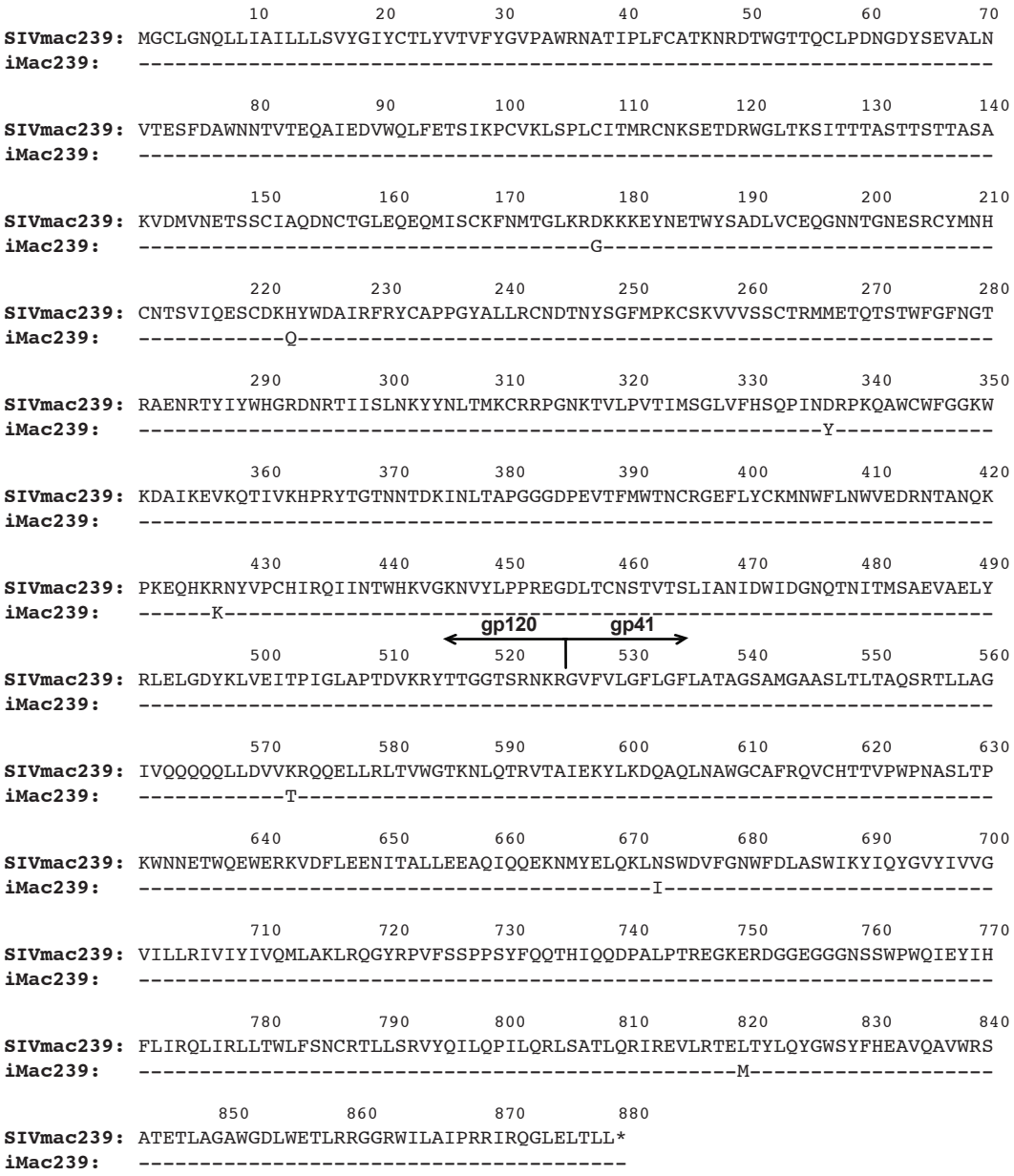


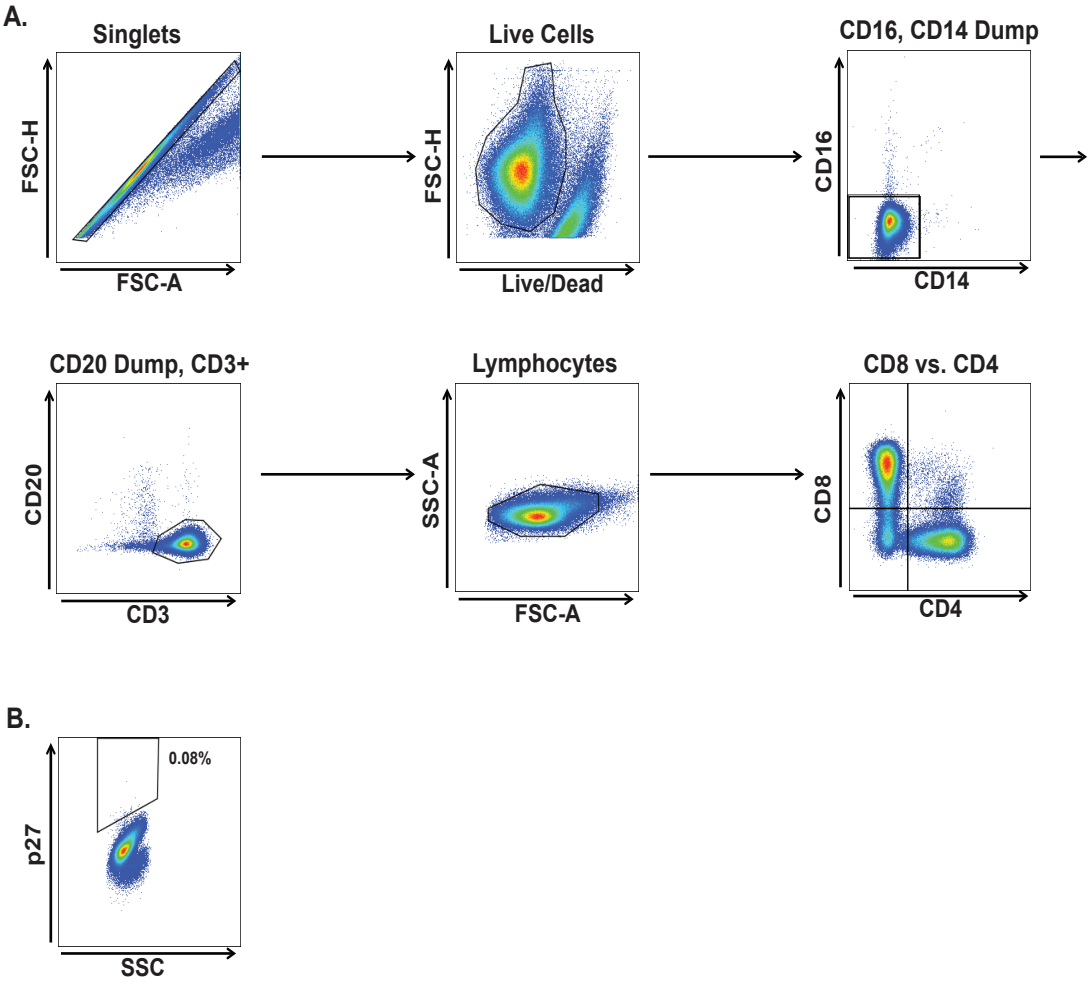
Supplemental Figure 2-1: Amino acid sequence alignment of HIV-1, HIV-2, and SIV gp120. Shown is a region that for HIV-1 contributes to the CD4 binding site. Aspartic acid-368 for HIV-1, which is critical for CD4 binding (i.e., D-385 for SIVmac239), is highlighted and highly conserved with the only exception being two SIVmnd isolates.



Supplemental Figure 2-2: Replication of SIVmac239 and iMac239 viruses containing Envs with full length or truncated cytoplasmic tails. Replication of viruses containing the iMac239 p8cl18 env clone with a truncated or full length cytoplasmic tail (CT) are shown in CD4+ Sup/RhR5 (Left panel) and CD4- BC7/RhR5 cells (Right panel). SIVs with parental SIVmac239 Envs with and without the CT truncation were used as controls. RT activity in culture supernatants was measured at the indicated time points. Results from a representative experiment are shown.



Supplemental Figure 2-3: Alignment of Env sequences for SIVmac239 and iMac239. Cleavage sites for gp120 and gp41 are indicated. Stop codons are denoted by asterisks (*). A stop codon at position 734 in the gp41 cytoplasmic tail, acquired as expected (76) during in vitro passaging of iMac239 in human T cell lines and present in the p8cl18 env clone, was repaired to create the iMac239 Env.



Supplemental Figure 2-4: Flow cytometry gating strategy for analysis of SIV-infected PBMCs. Monoclonal antibodies included those reactive with CD3, CD4, CD8, CD20, CD14, CD16 and SIV p27 Gag. (A) Gating strategy is shown for CD4+ and CD8+ T cells. Shown are cytograms for uninfected cells. **(B)** A representative cytogram of uninfected cells is shown to demonstrate negligible staining for p27 Gag.

References

1.**Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393**:648–59.

2. **Wyatt R, Sodroski J. 1998.** The HIV-1 Envelope Glycoproteins: Fusogens, Antigens, and Immunogens. Science **280**:1884–1888.

3. **Wilen CB, Tilton JC, Doms RW**. 2012. HIV: Cell Binding and Entry, p. 23–36. In HIV From Biology to Prevention and Treatment.

4. **Liu J, Bartesaghi A, Borgnia MJ, Sapiro G, Subramaniam S**. 2008. Molecular architecture of native HIV-1 gp120 trimers. Nature **455**:109–13.

5. **Mao Y, Wang L, Gu C, Herschhorn A, Desormeaux A, Finzi A, Xiang S-H, Sodroski JG**. 2013. Molecular architecture of the uncleaved HIV-1 envelope glycoprotein trimer. Proc Natl Acad Sci **110**:12438–12443.

6. **Myszka DG, Sweet RW, Hensley P, Brigham-Burke M, Kwong PD, Hendrickson WA, Wyatt R, Sodroski J, Doyle ML**. 2000. Energetics of the HIV gp120-CD4 binding reaction. Proc Natl Acad Sci **97**:9026–31.

7. **Park HE, Gruenke JA, White JM**. 2003. Leash in the groove mechanism of membrane fusion. Nat Struct Biol **10**:1048–53.

8. **Olshevsky U, Helseth E, Furman C, Li J, Haseltine W, Sodroski J**. 1990. Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. J Virol **64**:5701–7.

9. **Decker JM, Bibollet-Ruche F, Wei X, Wang S, Levy DN, Wang W, Delaporte E, Peeters M, Derdeyn CA, Allen S, Hunter E, Saag MS, Hoxie JA, Hahn BH, Kwong PD, Robinson JE, Shaw GM**. 2005. Antigenic conservation and immunogenicity of the HIV coreceptor binding site. J Exp Med **201**:1407–1419.

10. **Labrijn AF, Poignard P, Raja A, Zwick MB, Delgado K, Franti M, Binley J, Vivona V, Grundner C, Huang C-C, Venturi M, Petropoulos CJ, Wrin T, Dimitrov DS, Robinson J, Kwong PD, Wyatt RT, Sodroski J, Burton DR**. 2003. Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. J Virol **77**:10557–10565.

11. **Kalams SA, Walker BD**. 1998. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. J Exp Med **188**:2199–2204.

12. **Onabajo OO, Mattapallil JJ**. 2013. Expansion or Depletion of T Follicular Helper cells During HIV Infection: Consequences for B cell Responses. Curr HIV Res **11**:595–600.

13. **Heeney JL**. 2002. The critical role of CD4+ T-cell help in immunity to HIV. Vaccine **20**:1961–1963.

14. **Altfeld M, Rosenberg ES.** 2000. The role of CD4(+) T helper cells in the cytotoxic T lymphocyte response to HIV-1. Curr Opin Immunol **12**:375–80.

15. **Yamane H, Paul WE**. 2013. Early signaling events that underlie fate decisions of naive CD4+ T cells towards distinct T-helper cell subsets. Immunol Rev **252**:12–23.

16. **Zhu J, Paul WE**. 2010. Peripheral CD4 T cell differentiation regulated by networks of cytokines and transcription factors. Immunol Rev **238**:247–262.

17. **Ekkens MJ, Shedlock DJ, Jung E, Troy A, Pearce EL, Shen H, Pearce EJ**. 2007. Th1 and Th2 Cells Help CD8 T-Cell Responses. Infect Immun **75**:2291–2296.

18. **Klatt NR, Brenchley JM**. 2011. Th17 Cell Dynamics in HIV Infection. Curr Opin HIV AIDS **5**:135–140.

19. **Crotty S**. 2011. Follicular helper CD4 T cells (TFH). Annu Rev Immunol **29**:621–663.

20. **Liu X, Nurieva RI, Dong C**. 2013. Transcriptional regulation of folicular T-helper (Tfh) cells. Immunol Rev **252**:139–145.

21. **Linterman MA, Liston A, Vinuesa CG**. 2012. T-follicular helper cell differentiation and the co-option of this pathway by non-helper cells. Immunol Rev **247**:143–159.

22. **Vinuesa CG**. 2012. HIV and T follicular helper cells: a dangerous relationship. J Clin Invest **122**:3059–62.

23. **Mckinstry KK, Strutt TM, Swain SL**. 2010. Regulation of CD4+ T-cell contraction during pathogen challenge. Immunol Rev **236**:110–124.

24. **Bour-jordan H, Bluestone JA**. 2009. Regulating the regulators: costimulatory signals control the homeostatsis and function of regulatory T cells. Immunol Rev **229**:41–66.

25. **Brenchley JM, Paiardini M, Knox KS, Asher AI, Cervasi B, Asher TE, Scheinberg P, Price DA, Hage CA, Kholi LM, Khoruts A, Frank I, Else J, Schacker T, Silvestri G, Douek DC**. 2008. Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections. Blood **112**:2826–2835.

26. **Estes JD, Harris LD, Klatt NR, Tabb B, Pittaluga S, Paiardini M, Barclay GR, Smedley J, Pung R, Oliveira KM, Hirsch VM, Silvestri G, Douek DC, Miller CJ, Haase AT, Lifson J, Brenchley JM**. 2010. Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections. PLoS Pathog **6**(8): e1001052.doi:10.1371/journal.ppat.1001052.

27. **Endres MJ, Clapham PR, Marsh M, Ahuja M, Turner JD, McKnight A, Thomas JF, Stoebenau-Haggarty B, Choe S, Vance PJ, Wells TN, Power CA, Sutterwala SS, Doms RW, Landau NR, Hoxie JA**. 1996. CD4-independent infection by HIV-2 is mediated by fusin/CXCR4. Cell **87**:745–56.

28. **LaBranche CC, Hoffman TL, Romano J, Haggarty BS, Edwards TG, Matthews TJ, Doms RW, Hoxie JA**. 1999. Determinants of CD4 independence for a human immunodeficiency virus type 1 variant map outside regions required for coreceptor specificity. J Virol **73**:10310–9.

29. **Lin G, Lee B, Haggarty BS, Doms RW, Hoxie JA**. 2001. CD4-independent use of Rhesus CCR5 by human immunodeficiency virus Type 2 implicates an electrostatic interaction between the CCR5 N terminus and the gp120 C4 domain. J Virol **75**:10766–10778.

30. **Puffer BA, Pöhlmann S, Edinger AL, Carlin D, Sanchez MD, Reitter J, Watry DD, Fox HS, Desrosiers RC, Doms RW**. 2002. CD4 independence of simian immunodeficiency virus envs is associated with macrophage tropism, neutralization sensitivity, and attenuated pathogenicity. J Virol **76**:2595–2605.

31. **Puffer BA, Altamura LA, Pierson TC, Doms RW**. 2004. Determinants within gp120 and gp41 contribute to CD4 independence of SIV Envs. Virology **327**:16–25.

32. **Kolchinsky P, Mirzabekov T, Farzan M, Kiprilov E, Cayabyab M, Mooney LJ, Choe H, Sodroski J**. 1999. Adaptation of a CCR5-using, primary human immunodeficiency virus type 1 isolate for CD4-independent replication. J Virol **73**:8120–6.

33. **Kolchinsky P, Kiprilov E, Bartley P, Rubinstein R, Sodroski J**. 2001. Loss of a single N-Linked glycan allows CD4-Independent Human Immunodeficiency Virus Type 1 infection by altering the position of the gp120 V1 / V2 variable loops. J Virol **75**:3435–3443.

34. **Dumonceaux J, Nisole S, Chanel C, Quivet L, Amara A, Baleux F, Briand P, Hazan U**. 1998. Spontaneous mutations in the env gene of the human immunodeficiency virus type 1 NDK isolate are associated with a CD4-independent entry phenotype. J Virol **72**:512–9.

35. **Dumonceaux J, Goujon C, Joliot V, Briand P, Hazan U**. 2001. Determination of essential amino acids involved in the CD4-Independent tropism of the X4 Human Immunodeficiency Virus Type 1 m7NDK isolate : role of potential N glycosylations in the C2 and V3 regions of gp120. J Virol **75**:5425–5428.

36. **Boyd DF, Peterson D, Haggarty BS, Jordan APO, Hogan MJ, Goo L, Hoxie JA, Overbaugh J**. 2015. Mutations in HIV-1 envelope that enhance entry with the macaque CD4 receptor alter antibody recognition by disrupting quaternary interactions within the trimer. J Virol **89**:894–907.

37. **Hoffman TL, LaBranche CC, Zhang W, Canziani G, Robinson J, Chaiken I, Hoxie JA, Doms RW**. 1999. Stable exposure of the coreceptor-binding site in a CD4-independent HIV-1 envelope protein. PNAS **96**:6359–64.

38. **Edwards TG, Hoffman TL, LaBranche CC, Romano J, Adkinson J, Sharron M, Hoxie JA, Doms RW**. 2001. Relationships between CD4 independence , neutralization sensitivity , and exposure of a CD4-Induced epitope in a Human Immunodeficiency Virus Type 1 envelope protein. J Virol **75**:5230–5239.

39. **White TA, Bartesaghi A, Borgnia MJ, Meyerson JR, de la Cruz MJV, Bess JW, Nandwani R, Hoxie JA, Lifson JD, Milne JLS**. 2010. Molecular architectures of trimeric SIV and HIV-1 envelope glycoproteins on intact viruses : strain- dependent variation in quaternary structure. PLoS Pathog **6**(12): e1001249. doi:10.1371/journal.ppat.1001249.

40. **White TA, Bartesaghi A, Borgnia MJ, de la Cruz MJV, Nandwani R, Hoxie JA, Bess JW, Lifson JD, Milne JLS, Subramaniam S**. 2011. Three-Dimensional structures of soluble CD4-bound states of trimeric Simian Immunodeficiency Virus envelope glycoproteins determined by using cryo-electron tomography. J Virol **85**:12114–12123.

41. **Flaherty MT, Hauer DA, Mankowski JL, Zink MC, Clements JE**. 1997. Molecular and biological characterization of a neurovirulent molecular clone of simian immunodeficiency virus. J Virol **71**:5790–8.

42. **Mori K, Ringler DJ, Kodama T, Desrosiers RC**. 1992. Complex determinants of macrophage tropism in env of simian immunodeficiency virus. J Virol **66**:2067–75.

43. **Banapour B, Marthas ML, Ramos RA, Lohman BL, Unger RE, Gardner MB, Pedersen NC, Luciw PA**. 1991. Identification of viral determinants of macrophage tropism for simian immunodeficiency virus SIVmac. J Virol **65**:5798–5805.

44. **Ortiz AM, Klatt NR, Li B, Yi Y, Tabb B, Hao XP, Sternberg L, Lawson B, Carnathan PM, Cramer EM, Engram JC, Little DM, Ryzhova E, Gonzalez-scarano F, Paiardini M, Ansari AA, Ratcliffe S, Else JG, Brenchley JM, Collman RG, Estes JD, Derdeyn CA, Silvestri G**. 2011. Depletion of CD4 + T cells abrogates post-peak decline of viremia in SIV-infected rhesus macaques. J Clin Invest **121**:4433–45.

45. **Kolchinsky P, Kiprilov E**. 2001. Increased neutralization sensitivity of CD4-independent Human Immunodeficiency Virus variants. J Virol **75**:2041–2050.

46. **Francella N, Gwyn SE, Yi Y, Li B, Xiao P, Elliott STC, Ortiz AM, Hoxie JA, Paiardini M, Silvestri G, Derdeyn CA, Collman RG**. 2013. CD4+ T cells support production of Simian Immunodeficiency Virus env antibodies that enforce CD4-dependent entry and shape tropism in vivo. J Virol **87**:9719–9732.

47. **Francella N, Elliott ST, Yi Y, Gwyn SE, Ortiz AM, Li B, Silvestri G, Paiardini M, Derdeyn CA, Collman RG**. 2013. Decreased plasticity of coreceptor use by CD4-independent SIV Envs that emerge in vivo. Retrovirology **10**:133.

48. **Mori K, Rosenzweig M, Desrosiers RC**. 2000. Mechanisms for adaptation of simian immunodeficiency virus to replication in alveolar macrophages. J Virol **74**:10852–9.

49. **Mankowski JL, Flaherty MT, Spelman JP, Hauer DA, Didier PJ, Amedee AM, Murphey-Corb M, Kirstein LM, Muñoz A, Clements JE, Zink MC**. 1997. Pathogenesis of simian immunodeficiency virus encephalitis: viral determinants of neurovirulence. J Virol **71**:6055–60.

50. **Banapour B, Marthas ML, Munn RJ, Luciw PA**. 1991. In vitro macrophage tropism of pathogenic and nonpathogenic molecular clones of simian immunodeficiency virus (SIVmac). Virology **183**:12–19.

51. **Micci L, Alvarez X, Iriele RI, Ortiz AM, Ryan ES, McGary CS, Deleage C, McAtee BB, He T, Apetrei C, Easley K, Pahwa S, Collman RG, Derdeyn CA, Davenport MP, Estes JD, Silvestri G, Lackner AA, Paiardini M**. 2014. CD4 depletion in SIV-infected macaques results in macrophage and microglia infection with rapid turnover of infected cells. PLoS Pathog **10**(10): e1004467. doi:10.1371/journal.ppat.1004467.

52. **Joseph SB, Arrildt KT, Swanstrom AE, Schnell G, Lee B, Hoxie JA, Swanstrom R**. 2014. Quantification of entry phenotypes of macrophage-tropic HIV-1 across a wide range of CD4 densities. J Virol **88**:1858–1869.

53. **Gorry PR, Taylor J, Holm GH, Mehle A, Morgan T, Cayabyab M, Farzan M, Wang H, Bell JE, Kunstman K, Moore JP, Wolinsky SM, Gabuzda D**. 2002. Increased CCR5 affinity and reduced CCR5 / CD4 Dependence of a neurovirulent primary Human Immunodeficiency Virus Type 1 isolate. J Virol **76**:6277–6292.

54. **Li G-H, Anderson C, Jaeger L, Do T, Major EO, Nath A**. 2015. Cell-to-cell contact facilitates HIV transmission from lymphocytes to astrocytes via CXCR4. AIDS **29**:755–766.

55. **Bhattacharya J, Peters PJ, Clapham PR**. 2003. CD4-independent infection of HIV and SIV: implications for envelope conformation and cell tropism in vivo. AIDS **17**:S35–S43.

56. **Edinger AL, Mankowski JL, Doranz BJ, Margulies BJ, Lee B, Rucker J, Sharron M, Hoffman TL, Berson JF, Zink MC, Hirsch VM, Clements JE, Doms RW**. 1997. CD4-independent, CCR5-dependent infection of brain capillary endothelial cells by a neurovirulent simian immunodeficiency virus strain. Proc Natl Acad Sci **94**:14742–7.

57. **Reeves JD, Hibbitts S, Simmons G, McKnight A, Azevedo-Pereira JM, Moniz-Pereira J, Clapham PR**. 1999. Primary human immunodeficiency virus type 2 (HIV-2) isolates infect CD4-negative cells via CCR5 and CXCR4: comparison with HIV-1 and simian immunodeficiency virus and relevance to cell tropism in vivo. J Virol **73**:7795–804.

58. **Hoxie JA, Labranche CC, Endres MJ, Turner JD, Berson JF, Doms RW, Matthews TJ**. 1998. CD4-independent utilization of the CXCR4 chemokine receptor by HIV-1 and HIV-2. J Reprod Immunol **41**:197–211.

59. **Gallo SA, Puri A, Blumenthal R**. 2001. HIV-1 gp41 six-helix bundle formation occurs rapidly after the engagement of gp120 by CXCR4 in the HIV-1 Env-mediated fusion process. Biochemistry **40**:12231–6.

60. **Finzi A, Pacheco B, Xiang S-H, Pancera M, Herschhorn A, Wang L, Zeng X, Desormeaux A, Kwong PD, Sodroski J.** 2012. Lineage-specific differences between Human and Simian Immunodeficiency Virus regulation of gp120 trimer association and CD4 binding. J Virol **86**:8974–8986.

61. **Pancera M, Majeed S, Ban Y-EA, Chen L, Huang C, Kong L, Kwon Y Do, Stuckey J, Zhou T, Robinson JE, Schief WR, Sodroski J, Wyatt R, Kwong PD**. 2010. Structure of HIV-1 gp120 with gp41-interactive region reveals layered envelope architecture and basis of conformational mobility. Proc Natl Acad Sci **107**:1166–71.

62. **Li Y, Migueles SA, Welcher B, Svehla K, Phogat A, Louder MK, Wu X, Shaw GM, Connors M, Wyatt RT, Mascola JR**. 2007. Broad HIV-1 neutralization mediated by CD4-binding site antibodies. Nat Med **13**:1032–1034.

63. **Arthos J, Deen KC, Chaikin IM, Fornwald JA, Sathe G, Sattentau QJ, Clapham PR, Weiss RA, McDougal JS, Pietropaolo C**. 1989. Identification of the residues in human CD4 critical for the binding of HIV. Cell **57**:469–481.

64. **Wu H, Myszka DG, Tendian SW, Brouillette CG, Sweet RW, Chaiken IM, Hendrickson WA**. 1996. Kinetic and structural analysis of mutant CD4 receptors that are defective in HIV gp120 binding. Proc Natl Acad Sci **93**:15030–5.

65. **Ryzhova E, Whitbeck JC, Canziani G, Westmoreland SV, Cohen GH, Eisenberg RJ, Lackner A, González-Scarano F**. 2002. Rapid progression to simian AIDS can be accompanied by selection of CD4-independent gp120 variants with impaired ability to bind CD4. J Virol **76**:7903–7909.

66. **Del Prete GQ, Leslie GJ, Haggarty B, Jordan APO, Romano J, Hoxie JA**. 2010. Distinct molecular pathways to X4 tropism for a V3-truncated human immunodeficiency virus type 1 lead to differential coreceptor interactions and sensitivity to a CXCR4 antagonist. J Virol **84**:8777–89.

67. **Del Prete GQ, Haggarty B, Leslie GJ, Jordan APO, Romano J, Wang N, Wang J, Holmes MC, Montefiori DC, Hoxie JA**. 2009. Derivation and characterization of a simian immunodeficiency virus SIVmac239 variant with tropism for CXCR4. J Virol **83**:9911–22.

68. **Kestler H, Kodama T, Ringler D, Marthas M, Pedersen N, Lackner AA, Regier D, Sehgal P, Daniel M, King N.** 1990. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science **248**:1109–12.

69. **Rucker J, Doranz BJ, Edinger AL, Long D, Berson JF, Doms RW**. 1997. Cell-cell fusion assay to study the role of chemokine receptors in human immunodeficiency virus type 1 entry. Methods Enzymol **288**:118–133.

70. **Edinger AL, Doms RW**. 1999. A Cell-Cell fusion assay to monitor HIV-1 Env interactions with chemokine receptors. Methods Mol Med **17**:41–9.

71. **Montefiori DC**. 2005. Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. Curr Protoc Immunol Chapter 12:Unit 12.11.

72. **Edinger AL, Ahuja M, Sung T, Baxter KC, Haggarty B, Doms RW, Hoxie JA**. 2000. Characterization and epitope mapping of neutralizing monoclonal antibodies produced by immunization with oligomeric simian immunodeficiency virus envelope protein. J Virol **74**:7922–7935.

73. **Kong R, Li H, Georgiev I, Changela A, Bibollet-Ruche F, Decker JM, Rowland-Jones SL, Jaye A, Guan Y, Lewis GK, Langedijk JPM, Hahn BH, Kwong PD, Robinson JE, Shaw GM**. 2012. Epitope mapping of broadly neutralizing HIV-2 human monoclonal antibodies. J Virol **86**:12115–12128.

74. **Robinson JE, Cole KS, Elliott DH, Lam H, Amedee AM, Means R, Desrosiers RC, Clements J, Montelaro RC, Murphey-Corb M**. 1998. Production and characterization of SIV envelope-specific rhesus monoclonal antibodies from a macaque asymptomatically infected with a live SIV vaccine. AIDS Res Hum Retroviruses **14**:1253–1262.

75. **Cole KS, Alvarez M, Elliott DH, Lam H, Martin E, Chau T, Micken K, Rowles JL, Clements JE, Murphey-Corb M, Montelaro RC, Robinson JE**. 2001. Characterization of neutralization epitopes of simian immunodeficiency virus (SIV) recognized by rhesus monoclonal antibodies derived from monkeys infected with an attenuated SIV strain. Virology **290**:59–73.

76. **Robinson JE, Holton D, Pacheco-morell S, Liu J, McMurdo H**. 1990. Identification of Conserved and Variant Epitopes of Human Immunodeficiency Virus Type 1 (HIV-1) gp120 by Human Monoclonal Antibodies Produced by EBV-Transformed Cell Lines. AIDS Res Hum Retroviruses **6**:567–579.

77. **Kodama T, Wooley DP, Naidu YM, Kestler HW, Daniel MD, Li Y, Desrosiers RC**. 1989. Significance of premature stop codons in env of Simian Immunodeficiency Virus. J Virol **63**:4709–4714.

78. **Lock M, Greenberg ME, Lafrate AJ, Swigut T, Muench J, Kirchhoff F, Shohdy N, Skowronski J**. 1999. Two elements target SIV Nef to the AP-2 clathrin adaptor complex, but only one is required for the induction of CD4 endocytosis. EMBO J **18**:2722–2733.

79. **Chen BK, Gandhi R, Baltimore D**. 1996. CD4 down-modulation during Infection of human T cells with Human Immunodeficiency Virus Type 1 involves independent activities. J Virol **70**:6044–6053.

80. **Aiken C, Konner J, Landau NR, Lenburg ME, Trono D**. 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell **76**:853–864.

81. **Piguet V, Schwartz O, Le Gall S, Trono D**. 1999. The downregulation of CD4 and MHC-I by primate lentiviruses: a paradigm for the modulation of cell surface receptors. Immunol Rev **168**:51–63.

82. **Elliott STC, Wetzel KS, Francella N, Bryan S, Romero DC, Riddick NE, Shaheen F, Vanderford T, Derdeyn CA, Silvestri G, Paiardini M, Collman RG**. 2015. Dualtropic CXCR6/CCR5 Simian Immunodeficiency Virus (SIV) infection of sooty mangabey primary lymphocytes: distinct coreceptor use in natural versus pathogenic hosts of SIV. J Virol **89**:9252–9261.

83. **Puffer BA, Sharron M, Coughlan CM, Baribaud F, McManus CM, Lee B, David J, Price K, Horuk R, Tsang M, Doms RW**. 2000. Expression and coreceptor function of APJ for primate immunodeficiency viruses. Virology **276**:435–44.

84. **Rucker J, Edinger AL, Sharron M, Samson M, Lee B, Berson JF, Yi Y, Margulies B, Collman RG, Doranz BJ, Parmentier M, Doms RW**. 1997. Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses. J Virol **71**:8999–9007.

85. **Gautam R, Gaufin T, Butler I, Gautam A, Barnes M, Mandell D, Pattison M, Tatum C, Macfarland J, Monjure C, Marx PA, Pandrea I, Apetrei C**. 2009. Simian immunodeficiency virus SIVrcm, a unique CCR2-tropic virus, selectively depletes memory CD4+ T cells in pigtailed macaques through expanded coreceptor usage in vivo. J Virol **83**:7894–7908.

86. **Edinger AL, Hoffman TL, Sharron M, Lee B, Dowd BO, Doms RW**. 1998. Use of GPR1 , GPR15 , and STRL33 as coreceptors by diverse Human Immunodeficiency Virus Type 1 and Simian Immunodeficiency Virus envelope proteins. Pathology **249**:367–378.

87. **Deng HK, Unutmaz D, Kewal-Ramani VN, Littman DR**. 1997. Expression cloning of new receptors used by simian and human immunodeficiency viruses. Nature **388**:296–300.

88. **Vodros D, Thorstensson R, Doms RW, Fenyo EM, Reeves JD**. 2003. Evolution of coreceptor use and CD4-independence in envelope clones derived from SIVsm-infected macaques. Virology **316**:17–28.

89. **Johnston PB, Dubay JW, Hunter E**. 1993. Truncations of the simian immunodeficiency virus transmembrane protein confer expanded virus host range by removing a block to virus entry into cells. J Virol **67**:3077–3086.

90. **Kiene M, Marzi A, Urbanczyk A, Bertram S, Fisch T, Nehlmeier I, Gnirss K, Karsten CB, Palesch D, Münch J, Chiodi F, Pöhlmann S, Steffen I**. 2012. The role of the alternative coreceptor GPR15 in SIV tropism for human cells. Virology **433**:73–84.

91. **Margulies BJ, Hauer DA, Clements JE**. 2001. Identification and comparison of eleven rhesus macaque chemokine receptors. AIDS Res Hum Retroviruses **17**:981–6.

92. **Nyamweya S, Hegedus A, Jaye A, Rowland-Jones S, Flanagan KL, Macallan DC**. 2013. Comparing HIV-1 and HIV-2 infection: Lessons for viral immunopathogenesis. Rev Med Virol **23**:221–240.

93. **Clapham PR, McKnight A, Weiss RA**. 1992. Human immunodeficiency virus type 2 infection and fusion of CD4-negative human cell lines: induction and enhancement by soluble CD4. J Virol **66**:3531–3537.

94. **Musich T, Peters PJ, Duenas-Decamp MJ, Gonzalez-Perez MP, Robinson J, Zolla-Pazner S, Ball JK, Luzuriaga K, Clapham PR**. 2011. A conserved determinant in the V1 loop of HIV-1 modulates the V3 loop to prime low CD4 use and macrophage infection. J Virol **85**:2397–405.

95. **Luciw PA, Shaw KE, Unger RE, Planelles V, Stout MW, Lackner JE, Pratt-Lowe E, Leung NJ, Banapour B, Marthas ML**. 1992. Genetic and biological

comparisons of pathogenic and nonpathogenic molecular clones of simian immunodeficiency virus (SIVmac). AIDS Res Hum Retroviruses **8**:395–402.

96. **Reeves JD, Schulz TF**. 1997. The CD4-independent tropism of human immunodeficiency virus type 2 involves several regions of the envelope protein and correlates with a reduced activation threshold for envelope-mediated fusion. J Virol **71**:1453–1465.

97. **Yen P-J, Herschhorn A, Haim H, Salas I, Gu C, Sodroski J, Gabuzda D**. 2014. Loss of a conserved N-linked glycosylation site in the simian immunodeficiency virus envelope glycoprotein V2 region enhances macrophage tropism by increasing CD4-independent cell-to-cell transmission. J Virol **88**:5014–28.

98. **Sanders RW, Derking R, Cupo A, Julien J-P, Yasmeen A, de Val N, Kim HJ, Blattner C, de la Peña AT, Korzun J, Golabek M, de Los Reyes K, Ketas TJ, van Gils MJ, King CR, Wilson IA, Ward AB, Klasse PJ, Moore JP**. 2013. A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. PLoS Pathog **9**(9): e1003618. doi:10.1371/journal.ppat.1003618.

99. **Lyumkis D, Julien J-P, de Val N, Cupo A, Potter CS, Klasse PJ, Burton DR, Sanders RW, Moore JP, Carragher B, Wilson IA, Ward AB**. 2013. Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 Env trimer. Science **342**:1484–1490.

100. **Julien J-P, Cupo A, Sok D, Stanfield RL, Lyumkis D, Deller MC, Klasse P-J, Burton DR, Sanders RW, Moore JP, Ward AB, Wilson IA**. 2013. Crystal structure of a soluble cleaved HIV-1 envelope trimer. Science **342**.

101. **Kwon YD, Finzi A, Wu X, Dogo-Isonagie C, Lee LK, Moore LR, Schmidt SD, Stuckey J, Yang Y, Zhou T, Zhu J, Vicic DA, Debnath AK, Shapiro L, Bewley CA, Mascola JR, Sodroski JG, Kwong PD**. 2012. Unliganded HIV-1 gp120 core structures assume the CD4-bound conformation with regulation by quaternary interactions and variable loops. Proc Natl Acad Sci **109**:5663–5668.

102. **Haim H, Strack B, Kassa A, Madani N, Wang L, Courter JR, Princiotto A, McGee K, Pacheco B, Seaman MS, Smith AB, Sodroski J**. 2011. Contribution of intrinsic reactivity of the HIV-1 envelope glycoproteins to CD4-independent infection and global inhibitor sensitivity. PLoS Pathog **7**(6): e1002101. doi:10.1371/journal.ppat.1002101.

103. **Haim H, Si Z, Madani N, Wang L, Courter JR, Princiotto A, Kassa A, DeGrace M, McGee-Estrada K, Mefford M, Gabuzda D, Smith AB, Sodroski J**. 2009. Soluble CD4 and CD4-mimetic compounds inhibit HIV-1 infection by induction of a short-lived activated state. PLoS Pathog **5**(4): e1000360. doi:10.1371/journal.ppat.1000360.

104. **Courter JR, Madani N, Sodroski J, Schön A, Freire E, Kwong PD, Hendrickson WA, Chaiken IM, LaLonde JM, Smith AB**. 2014. Structure-based design, synthesis and validation of CD4-mimetic small molecule inhibitors of HIV-1 entry: conversion of a viral entry agonist to an antagonist. Acc Chem Res **47**:1228–37.

105. **Kamanna K, Aneja R, Duffy C, Kubinski P, Rodrigo MD, Bailey LD, McFadden K, Schon A, Holmes A, Tuzer F, Contarino M, Freire E, Chaiken IM**. 2013. Non-natural peptide triazole antagonists of HIV-1 envelope gp120. Chem Med Chem **8**:doi:10.1002/cmdc.201200422.

106. **Munro JB, Gorman J, Ma X, Zhou Z, Arthos J, Burton DR, Koff WC, Courter JR, Iii ABS, Kwong PD, Blanchard SC, Mothes W**. 2014. Conformational dynamics of single HIV-1 envelope trimers on the surface of native virions. Science **346**:759–763.

107. **Mattapallil JJ, Douek DC, Hill B, Nishimura Y, Martin M, Roederer M**. 2005. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. Nature **434**:1093–1097.

108. **Paiardini M, Cervasi B, Reyes-Aviles E, Micci L, Ortiz AM, Chahroudi A, Vinton C, Gordon SN, Bosinger SE, Francella N, Hallberg PL, Cramer E, Schlub T, Chan ML, Riddick NE, Collman RG, Apetrei C, Pandrea I, Else J, Munch J, Kirchhoff F, Davenport MP, Brenchley JM, Silvestri G**. 2011. Low levels of SIV infection in sooty mangabey central memory CD4+ T cells are associated with limited CCR5 expression. Nat Med **17**:830–6.

109. **Riddick NE, Hermann EA, Loftin LM, Elliott ST, Wey WC, Cervasi B, Taaffe J, Engram JC, Li B, Else JG, Li Y, Hahn BH, Derdeyn CA, Sodora DL, Apetrei C, Paiardini M, Silvestri G, Collman RG**. 2010. A novel CCR5 mutation common in Sooty Mangabeys reveals SIVsmm infection of CCR5-null natural hosts and efficient alternative coreceptor use in vivo. PLoS Pathog **6**(8): e1001064. doi:10.1371/journal.ppat.1001064.

110. **Pandrea I, Apetrei C, Gordon S, Barbercheck J, Dufour J, Bohm R, Sumpter B, Roques P, Marx PA, Hirsch VM, Kaur A, Lackner AA, Veazey RS, Silvestri G**. 2008. Paucity of CD4+ CCR5+ T cells is a typical feature of natural SIV hosts. Blood **109**:1069–1076.

111. **Veazey RS, DeMaria M, Chalifoux L V, Shvetz DE, Pauley DR, Knight HL, Rosenzweig M, Johnson RP, Desrosiers RC, Lackner AA**. 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. Science **280**:427–431.

112. **Ling B, Veazey RS, Luckay A, Penedo C, Xu K, Lifson JD, Marx PA**. 2002. SIV(mac) pathogenesis in rhesus macaques of Chinese and Indian origin compared with primary HIV infections in humans. AIDS **16**:1489–96.

113. **Klatt NR, Harris LD, Vinton CL, Sung H, Briant JA, Morcock D, Mcginty JW, Lifson JD, Lafont BA, Martin A, Levine AD, Estes JD, Brenchley JM**. 2010. Compromised gastrointestinal integrity in pigtail macaques is associated with increased microbial translocation, immune activation and IL-17 production in the absence of SIV infection. Mucosal Immun **3**:387–398.

114. **Brenchley JM**. 2013. Mucosal immunity in human and simian immunodeficiency lentivirus infections. Mucosal Immunol **6**:657–65.

CHAPTER 3

Pathogenesis and Immunogenicity of a Novel Variant of SIVmac239 Lacking CD4 Tropism

Abstract

CD4 tropism of primate lentiviruses has been proposed to disrupt host antiviral immune responses and to contribute to persistent viral infection. To explore how immune responses might be different in the context of a CD4-sparing infection, we derived a CD4-independent, CCR5-tropic variant of SIVmac239 that lacked a functional CD4 binding site. This virus, iMac239-ΔD385, was inoculated into four rhesus macaques lacking controlling MHC alleles. iMac239-ΔD385 generated a robust acute infection, however $CD4^+$ T cell populations in blood and gut remained stable. Following the acute peak, plasma RNA rapidly decreased to <100 copies/ml, correlating with the appearance of high and persisting neutralizing antibody titers to Tier-1 SIV strains. At ≥300 dpi iMac239-ΔD385-infected animals and four naïve, Trim5α-matched controls were challenged intrarectally, weekly for up to ten weeks with SIVsmE660. All four controls became infected by challenge 2; three iMac239-ΔD385-infected animals became infected (two at challenge 2, one at challenge 7), and one remained uninfected. When factored by Trim5α, iMac239-ΔD385 animals exhibited a log reduction in peak SIVsmE660 viral loads compared to their matched controls. Three out of four E660-infected controls lost $CD4^+$ T-cells in lamina propria, while two of three SIVsmE660-infected iMac239-ΔD385 animals maintained normal $CD4^+$ T cells up to 10 weeks post infection. Finally, two of three SIVsmE660-infected iMac239-ΔD385 animals exhibited an expansion of breadth and increase in magnitude of their humoral response post challenge and were able neutralize 100% of a Tier-2 SIVsmE660 strain. As the first model of a non-CD4 tropic primate lentiviral infection, this system may provide new insights into qualitative and/or

quantitative augmentation of host anti-viral immune responses and perhaps serve as a novel prime for immunization protocols.

Introduction

Pathogenic simian immunodeficiency virus infection of non-natural primate hosts recapitulates many of the characteristics of human immunodeficiency virus type 1 (HIV-1) infection in humans (1, 2) and thus is a valuable model in which to study the effects of various perturbations to the virus-host interaction. Similar to HIV-1, SIV utilizes the CD4 and CCR5 receptors for entry into $CD4^+$ T cells (3–5). During the acute phase of pathogenic infection, the host experiences high plasma viral loads that coincides with a sharp decline in $CD4^+$ $CCR5^+$ T cells in the gut-associated lymphoid tissue (GALT) (6–8). As the host adaptive immune system develops SIV specific responses, the viral load drops to a set point level that is lower than the acute phase, and this is maintained throughout the chronic phase of infection, contributing to a persistent state of immune activation (9–11). Notably, the magnitude of the set point can be influenced by certain host factors, including MHC Class I alleles (12–14). Although peripheral $CD4^+$ T cells do not undergo the dramatic depletion seen in the GALT, they gradually decline throughout the chronic phase until the ability of the host to regenerate new $CD4^+$ T cells is exhausted, resulting in the collapse of the immune system and development of clinical AIDS (15, 16). A hallmark feature of HIV-1 infection of humans and SIV infection of non-natural hosts is the inability of the host immune response, cellular or humoral, to outpace viral evolution and control viral replication.

The critical role of $CD4^+$ T cells as mediators of adaptive immunity means that their depletion during HIV-1 and SIV infection has dire consequences for the host and its ability to mitigate pathogenesis. Various subsets of $CD4^+$ T cells contribute to both arms of adaptive immunity, including Th1 cells that stimulate $CD8^+$ CTL development and

memory, Th17 cells that maintain the protective barrier at mucosal surfaces, T follicular helper cells (Tfh) that contribute to B cell development, and T-regulatory cells (17–24). Recent work has highlighted that Tfh cells are infected in large numbers, resulting in functional disruptions that have been implicated in B cell dysfunction (25–30). Additionally, it has been suggested that Tfh cells within germinal centers may serve as a viral reservoir (31) in addition to resting memory $CD4^+$ cells and macrophages (32–34). Irrespective of viral infection of $CD4^+$ T cells and the resulting consequences, the binding between the viral gp120 protein and CD4 has the potential to block MHC-II interactions with antigen presenting cells while also generating aberrant signaling, further disrupting CD4 dependent immune responses (35–37). Therefore, the unique ability of primate lentiviruses to bind the CD4 molecule, thereby targeting infection to CD4 cells could serve many roles in sustaining viral replication while simultaneously disrupting host antiviral immune responses.

While the majority of primate lentiviruses studied are CD4 tropic, there are a few exceptions. Previously described CD4-independent (CD4i) SIVs were originally identified as macrophage tropic strains and were then tested for their ability to enter cells with low levels, or in the absence, of CD4 in vitro (38–41). The extent to which these viruses could use the coreceptor alone for entry was largely assessed in the context of Env only based assays, including cell-cell fusion and pseudotype entry assays (39, 41, 42) in which Envs often had a truncated cytoplasmic tail as a result of passaging in human T cell lines (43). The presence of this tail truncation is significant because it results in an increase of Env surface expression (44), thereby increasing overall rates of entry and enhancing CD4-independence (43, 45, 46). Rarely were these Envs tested for CD4-

independent replication as infectious molecular clones in CD4-negative cell lines. It is also important to note that based on sequence analysis, the previously described CD4i SIV Envs have intact CD4 binding sites and thus presumably will still bind CD4 and utilize it for entry if it is available (40, 47, 48). Additionally, it has been shown that a CD4-independent HIV-1 strain exhibits faster fusion kinetics in the presence of CD4, suggesting that this class of virus, while capable of CD4-independent entry, will continue to use CD4 for increased entry efficiency (49).

We previously derived a CD4-independent variant of the pathogenic CD4-dependent, CCR5-tropic SIVmac239 (Chapter 2). This novel CD4i variant, termed iMac239-ΔD385, can efficiently use rhesus CCR5 as a sole mean of entry in a CD4-negative cell line and is also able to infect $CD4^-$ $CD8^+$ T cells in RhPBMC cultures. We identified a minimum set of Env mutations that impart CD4-independence and showed that a single mutation in gp120 was able to confer CD4-independent fusion to the SIVmac239 Env, but that this mutation in the context of a replication competent viral clone resulted in a severe fitness defect, suggesting a disrupted trimer conformation. We have also shown that this Env, unlike SIVmac239, is sensitive to neutralization by monoclonal antibodies targeting epitopes across gp120. Finally, our data showed that the iMac239-ΔD385 Env is insensitive to inhibition by sCD4, suggesting that binding of this Env is significantly reduced or ablated, making this a truly CD4-independent strain.

To study how the interactions between the virus and the host might be altered in the context of a CD4-sparing infection, we inoculated four rhesus macaques that were negative for MHC I controlling alleles with molecularly cloned iMac239-ΔD385. We observed that in all four animals acute replication of iMac239-ΔD385 was similar to

what has been reported for SIVmac239, however there was a delay to the time of acute peak (50–53). In tissues, we observed an expansion of cell targets beyond $CD3^+$ T cells, including an as yet unidentified cell type, and infection of macrophages at least two weeks earlier that what has been reported for SIVmac239 infection in vivo (54). Additionally we found that iMac239-ΔD385 infected cells were predominately located in the medulla, rather than the cortex, of the lymph node during the acute phase. Somewhat surprisingly, although macrophage tropic strains capable of CD4-independent infection are often associated with viral replication in the brain and central nervous system (CNS) pathologies (41, 55–57), we did not observe significant viral load in the cerebrospinal fluid (CSF) or any signs of clinical neuropathies in the four animals.

After the acute peak, all four animals quickly controlled iMac239-ΔD385 replication to undetectable levels; this viral control was sustained for up to 600 days post infection. All animals maintained their $CD4^+$ and $CD8^+$ T cells in the periphery and lamina propria throughout the acute and chronic phases of infection. Utilizing single genome amplification (SGA) we amplified and sequenced 30 viral amplicons from each animal at peak viremia and showed that in all four animals the mutations in the inoculum were maintained and in three of four animals no additional mutations became fixed in the viral swarm. All animals displayed high and sustained neutralizing antibody responses as well as polyfunctional $CD4^+$ and $CD8^+$ T cell responses.

Based on the robust immune responses garnered by iMac239-ΔD385 infection, we tested whether these responses would be sufficient to protect the animals from a pathogenic challenge. After weekly intrarectal inoculations with SIVsmE660 we observed that while all four naïve control animals were infected by the second challenge,

two iMac239-ΔD385 animals were infected on the second challenge, one on the seventh challenge, and one remained uninfected by SIVsmE660 after ten challenges. iMac239-ΔD385 displayed a trend towards lower acute viral peaks and delayed time to peak, however this study was not powered for statistical significance. Strikingly, two of three SIVsmE660 infected iMac239-ΔD385 animals displayed an enhanced humoral response after the challenge, resulting in the first reported case of 100% neutralization of the SIVsmE660.2A5-IAKN strain.

Taken together these results, albeit with small numbers of animals, indicate that SIV Env binding to CD4 is not required for robust in vivo replication and that $CD4^+$ T cells can, in fact, be spared during the course of infection. Additionally, these findings suggest that by redirecting viral targeting of $CD4^+$ T cells, the host is able to mount immune responses that are able to control viral replication of a novel variant of SIVmac239. While these responses were ultimately not able to protect against a pathogenic challenge in three of four animals, animals with favorable Trim5α alleles appeared to have an improved outcome post-challenge compared to matched controls, and the primary iMac239-ΔD385 infection may have served as a novel prime to improve humoral responses post challenge.

Methods

Animals, Viral Inoculations, and Sample Collection

(Pyone Aye, Andrew Lackner, TNPRC)

Four rhesus macaques were inoculated intravenously (i.v.) with 300 $TCID_{50}$ iMac239-ΔD385 (n=4). These four animals, along with four naïve rhesus macaques were

subsequently inoculated intrarectally (i.r.) with 5,000 $TCID_{50}$ SIVsmE660 (n=8). All animals were negative for MHC I alleles Mamu A*01, B*08, and B*17 and were maintained at the Tulane National Primate Research Center (TNPRC). The iMac239-ΔD385 viral stock was produced in 293T cells transfected with plasmids containing full-length proviral DNA. The SIVsmE660 stock was a viral swarm supplied by Dr. Vanessa Hirsch and was previously passaged in pigtail macaque PBMCs (58). Viruses were quantified by determining $TCID_{50}$ on rhesus macaque PBMCs. Prior to use, all animals tested negative for antibodies to SIV, STLV and Type D retrovirus and by PCR for Type D retrovirus. Animals were anesthetized with ketamine hydrochloride or isoflurane for collection of multiple blood samples, small intestinal biopsies (endoscopic duodenal pinch biopsies or jejunal resection biopsies) Animals were euthanized if they exhibited a loss of more than 25% of maximum body weight, anorexia for more than 4 days or major organ failure or medical conditions unresponsive to treatment.

All animals were maintained at TNPRC in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Research Council. The TNPRC Institutional Animal Care and Use Committee approved all studies.

Infection of PBMCs

Purified peripheral blood mononuclear cells (PBMCs) from rhesus macaques stored at -140°C were thawed and stimulated for 3 days with 5 μg/mL ConA at a concentration of 106 cells/mL in RPMI Complete media. Cells (5 x 106) were then inoculated with viruses (125 ng of p27 Gag) and media supplemented with IL-2 (100 IU/mL). After 24hrs, cells

were washed to remove the viral inoculum and cultured in fresh RPMI Complete media supplemented with IL-2 (100 IU/mL). Viral replication was monitored by p27 Gag expression.

Quantification of viral load in plasma

(Yuan Li, Mike Piatak, Jeff Lifson, NCI)

Plasma viral loads were determined at various times using a reverse transcription-polymerase chain reaction (RT-PCR) assay with a limit of detection between 15 and 60 SIV RNA copies/mL (59). To discriminate between iMac239-ΔD385 and SIVsmE660 replication post challenge *gag* sequence analysis was performed as previously described (60).

Cell-associated SIV DNA and RNA

(Yuan Li, Mike Piatak, Jeff Lifson, NCI)

Total DNA and RNA was extracted from lymph node mononuclear cell specimens, and DNA-PCR or RT-PCR for SIV *gag* sequences was performed as previously described (60).

Lymphocyte isolation from intestinal tissues

(Faith Schiro, Pyone Aye, TNPRC)

Intestinal cells were collected by endoscopic pinch or jejunal resection biopsies of the small intestine and isolated using EDTA/collagenase digestion and percoll density-gradient centrifugation as previously described (61–64).

Immunophenotyping of cells

(Workineh Torben, Bapi Pahar, TNPRC)

Immunophenotyping of cells was performed on isolated lamina propria lymphocytes (LPLs) and anti-coagulated whole blood using antibodies reactive with: CD3 (SP34), CD8 (SK1 or SK2), CD4 (L200), and CCR5 (3A9) all from BD Biosciences, San Jose CA. Viability was determined using Live/Dead stain (L34957) (Life Technologies, Grand Island, NY). Protocols for performing immunophenotyping have been described previously (65). In brief, cells were gated first on singlets, lymphocytes, followed by live cells and then on $CD3^+$ T cells and subsequently on $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cell subsets. Flow cytometry data were analyzed using Flowjo Software version 9.1 (TreeStar Inc., Ashland, OR).

Intracellular Cytokine Analysis

(Workineh Torben, Bapi Pahar, TNPRC)

Intracellular cytokine analysis of SIV-specific $CD4^+$ and $CD8^+$ peripheral blood and lamina propria lymphocytes was performed on all four iMac239-ΔD385 infected animals at various time points, as previously described (66, 67). Briefly, cells were stimulated with SIV peptides (Env, Gag, and Pol, NIH AIDS Research & Reference Reagent Program) and then brefeldin A (Sigma) was added. Media was used as a negative control and PMA/Ionomycin was used as a positive control. Cells were stained with anti-CD3 (SP34), -CD4 (L200), -CD8 (5H10) and a viability stain (L34957), then washed, permeabilized and stained with antibodies to: IL2 (MQ1-17H12), IFNγ (4S.B3), TNFα (MA611), CD107α (B-T47), and IL17 (eBio64DEC17) (BD Biosciences, San Jose, CA).

Cells were fixed in stabilizing fixative buffer (BD Biosciences, San Jose, CA) and analyzed on a Becton Dickinson LSR II flow cytometer.

The gating strategy was similar to the immunophenotyping described above. The percentages of CD107α, IL17, IFNγ, TNFα, and IL2 positive responses in each subset as well as negative or positive controls were assessed using Flowjo software, version 9.1 (TreeStar).

$CD8^+$ T cell depletion

(Pyone Aye, Andrew Lackner, TNPRC)

$CD8^+$ T cell depletion protocols were performed on two iMac239-ΔD385 infected animals (IC30 and II40) at 300 days post infection. Animals received the anti-CD8 antibody MT807R1, provided by the Nonhuman Primate Reagent Resource (Boston, MA) (68). Antibody was administered subcutaneously (s.c.) at 10mg/kg on day 0 and i.v. at 5mg/kg on days 3, 7, and 10. $CD8^+$ T cells in blood were monitored by flow cytometry, as described above.

MHC and Trim5α typing of rhesus macaques

(Pyone Aye, Andrew Lackner, TNPRC)

MHC class I genotyping was carried out by the Rhesus Macaque MHC Typing Core facility at TNPRC. The Trim5α genotypes of the eight rhesus macaques were determined by Dr. Welkin Johnson (Boston College) as previously described (69).

Assays for neutralizing antibodies

(Celia LaBranche, David Montefiori, Duke Central Reference Laboratory)

Neutralizing antibody responses were determined on pseudotyped viruses produced in 293T cells (70) using TZM-bl cells expressing CD4 and CCR5. All assays were conducted in triplicate as described previously (71). Viral Envs used in the neutralization panel included SIVmac251.6 (72), SIVsmE660/BR-CG7G-IR1(73, 74), SIVsmE660-2A5.VTRN (neutralization sensitive), and SIVsmE660-2A5.IAKN (neutralization resistant)(75).

Confocal Microscopy

(Xavier Alvarez-Hernandez, TNPRC)

Double confocal microscopy was performed to colocalize SIV p27 Gag protein with cell type-specific markers to determine the immunophenotype of infected cells, as previously described (76). Immunofluorescent labeling of T cells (rabbit polyclonal to CD3, Biocare Medical, Concord, CA, CD2), macrophages (mouse IgG_1 monoclonal to CD68, Dako, CD163, LN5), dendritic cells (CD123, CD209), and B cells (CD20). After incubation with the primary antibodies (anti-CD3, anti-CD68, or anti-p27) and subsequent washes, the appropriate species-specific secondary antibodies were applied; AlexaFluor 488 or 633 conjugated goat anti-rabbit or goat anti-mouse IgG_1 (Invitrogen, Carlsbad, CA). Confocal microscopy was performed using the sequential mode to capture separately the fluorescence from the different fluorochromes (Leica Microsystems, Exton, PA). NIH Image v1.62 and Adobe Photoshop v7 software were used to correct the colors collected in the different channels: Alexa 488 (green, Alexa 568 (red), Alexa 633 (blue) and differential interference contrast (DIC; grey scale).

Single Genome Amplification (SGA) Analysis

(A. Swanstrom; Brandon Keele, NCI)

SGA analyses were performed on plasma samples from all four iMac239-ΔD385 infected animals at 17 dpi. The entire *env* gene was sequenced using a limiting dilution PCR to insure that only one amplifiable molecule was present in each reaction, as previously described (65, 74). Sequence alignments were generated with the Macvector software and presented as highlighter plots (www.hiv.lanl.gov).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism v5.0c (GraphPad Software, Inc, La Jolla, CA).

Results

Infection of rhesus macaques with iMac239-ΔD385

Four rhesus macaques that were negative for MHC I controlling alleles (Mamu A*01, B*08, B*17) (12–14) were intravenously inoculated with a 300 $TCID_{50}$ dose of iMac239-ΔD385 or iMac239-ΔD385 Q739 (2 animals each). The Q739 mutation occurs in the cytoplasmic tail of iMac239-ΔD385 Env, and while it is a noncoding change in Env, it results in an Arg to Lys change in the second exon of Tat (at AA position 103) and a Gly to Arg change in the second exon of Rev (at AA position 29). There was no obvious effect of this mutation on in vitro replication in rhesus PBMCs (Suppl. Fig. 3-1). However, in the interest of studying a model in which any changes in phenotype are due

only to alterations in Env, we inoculated two rhesus macaques with this corrected virus, iMac239-ΔD385 Q739 (HV24 and IK16) in addition to two animals infected with iMac239-ΔD385 (IC30 and II40).

All four animals exhibited an acute plasma viral peak of 10^5-10^7 copies vRNA/mL (Fig. 3-1). The two animals infected with iMac239-ΔD385 Q739 had the lowest acute peak viral load, however given the small number of animals in this study it is unclear whether this was a result of the correction of the mutation or natural variability between rhesus macaques. In all subsequent analyses there were no overt differences between iMac239-ΔD385 and iMac239-ΔD385 Q739 infected animals. The acute viral peak in all animals occurred at 17 days post infection (dpi), which is delayed from the historical peak of SIVmac239, usually occurring at 10-14 dpi (50–53). Following the acute peak, all four animals quickly controlled viral replication to 10^2-10^3 vRNA copies/mL by 42 dpi, and by 150 dpi all animals had controlled plasma virus to undetectable levels (<60 vRNA copies/mL). This viral control was maintained throughout chronic infection (≥300 dpi) (Fig. 3-1). These data indicate that while this novel CD4-independent strain is fit and is capable of replicating acutely at wildtype levels, the host immune response is able to quickly control viral replication.

Evolution of iMac239-ΔD385 in vivo

We considered the possibility that the iMac239-ΔD385 Env may evolve in vivo to improve its fitness, regain a functional CD4 binding site, or evade immune pressure given its neutralization sensitivity. To test this hypothesis, we performed single genome amplification and sequencing on plasma viral RNA at peak viral infection at 17 dpi. In

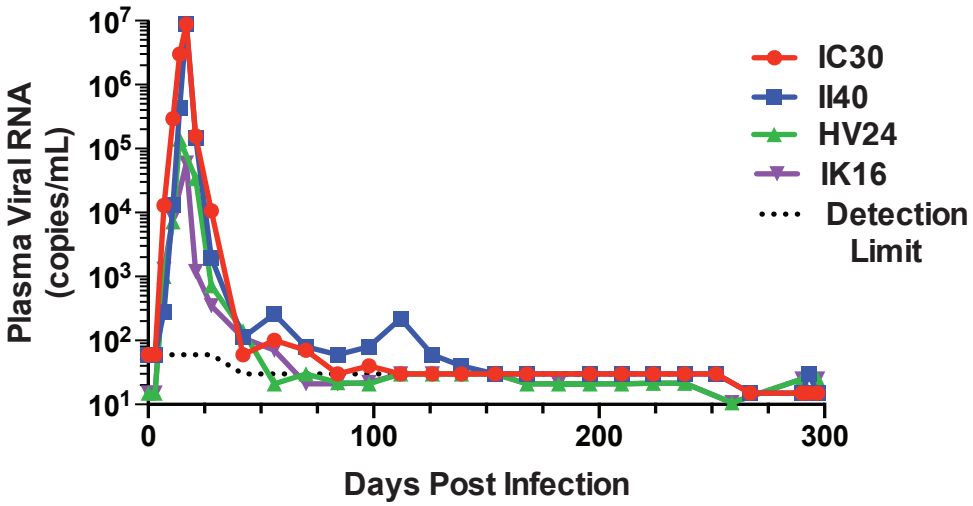


Figure 3-1: Viral loads in iMac239-ΔD385 infected rhesus macaques. Plasma viral RNA levels were measured using RT-PCR. The first 300 days of infection are shown.

three of four animals there was no consensus evolution of the viral Env (Fig. 3-2). The few mutations that were observed appeared to be random or due to APOBEC3G (reviewed in (77)) and none of them became fixed in the viral population. The fourth animal, IK16, showed fixation of a single mutation in 40% of the 30 viral clones sampled (Fig. 3-2). This mutation, L782P, occurs within a putative MHC Class II epitope for the MHC II allele DRBW*201 (78). MHC II genotyping of this macaque revealed that it carried the DRBW*201 allele. The presence of this allele, coupled with the observation that this animal had the lowest acute viral load (10^5 vRNA copies/mL, Fig. 3-1) of the four animals suggested that this animal was exerting immune pressure on the virus through an MHC Class II mechanism. Further investigation is required to assess this possibility.

Our sequencing analysis revealed that all four animals retained the original iMac239-ΔD385 mutations, including the ΔD385 within the CD4 binding site. This suggests that the inoculum was capable of robust in vivo replication and that the virus did not reacquire CD4 dependence during the acute phase.

$CD4^+$ and $CD8^+$ T cell population dynamics in iMac239-ΔD385 infected animals

Given that we believed iMac239-ΔD385 would not selectively target $CD4^+$ T cells, it was important to monitor the $CD4^+$ T cell populations in the periphery and lamina propria. All four animals maintained their $CD4^+$ T cell populations in both compartments with no significant loss of $CD4^+$ T cells (Fig. 3-3A). This is in striking contrast to SIVmac239 infection in which animals typically lose up to 90% of $CD4^+$ T cells in the lamina propria during the acute phase (7, 65, 79).

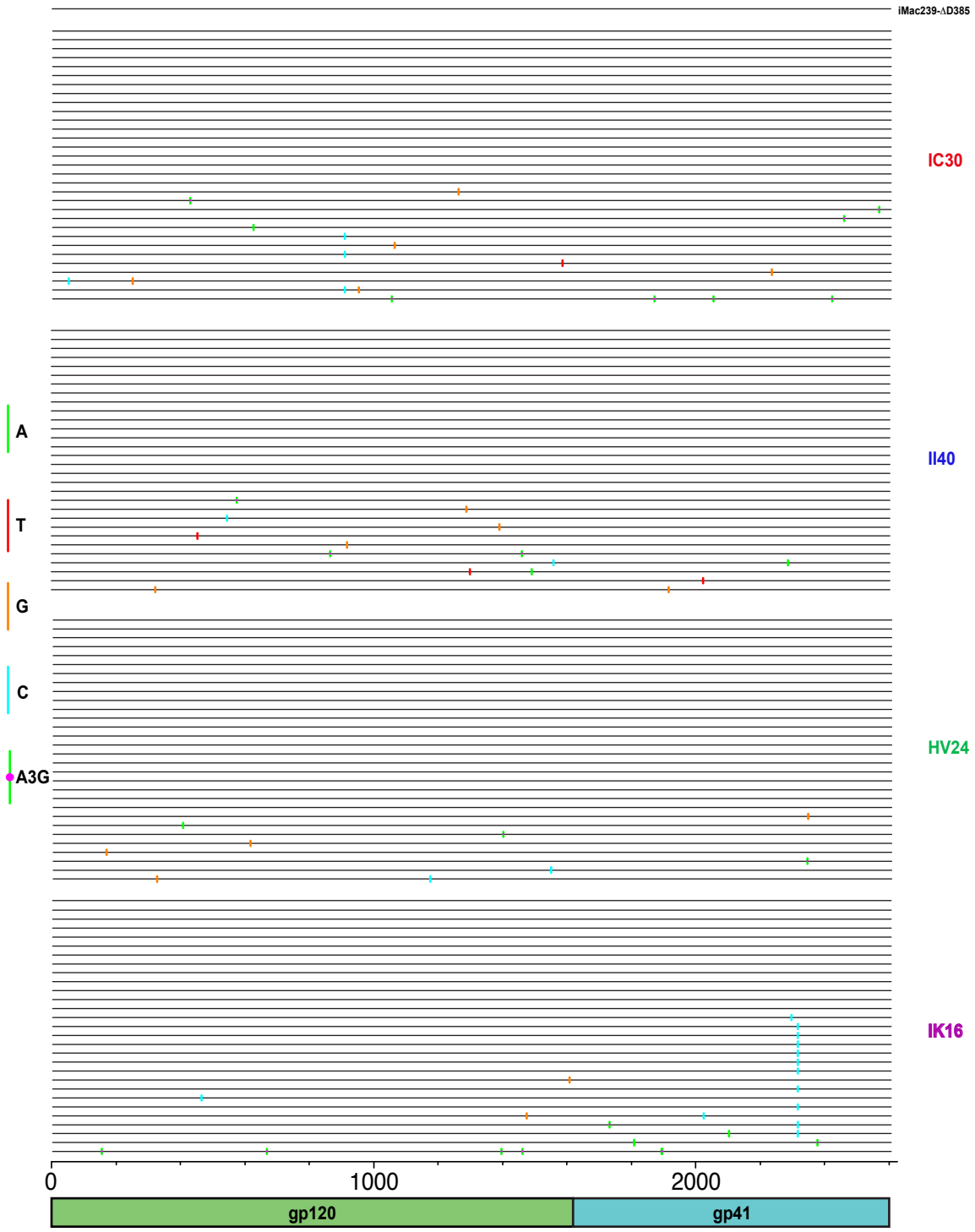


Figure 3-2: Viral evolution of *envs* from iMac239-ΔD385 infected rhesus macaques. Highlighter nucleotide sequence analysis (www.hiv.lanl.gov) for animals IC30, II40, HV24, and IK16. Highlighter sequence plots show individual nucleotide polymorphisms between each amplicon sequence and the iMac239-ΔD385 reference sequence.

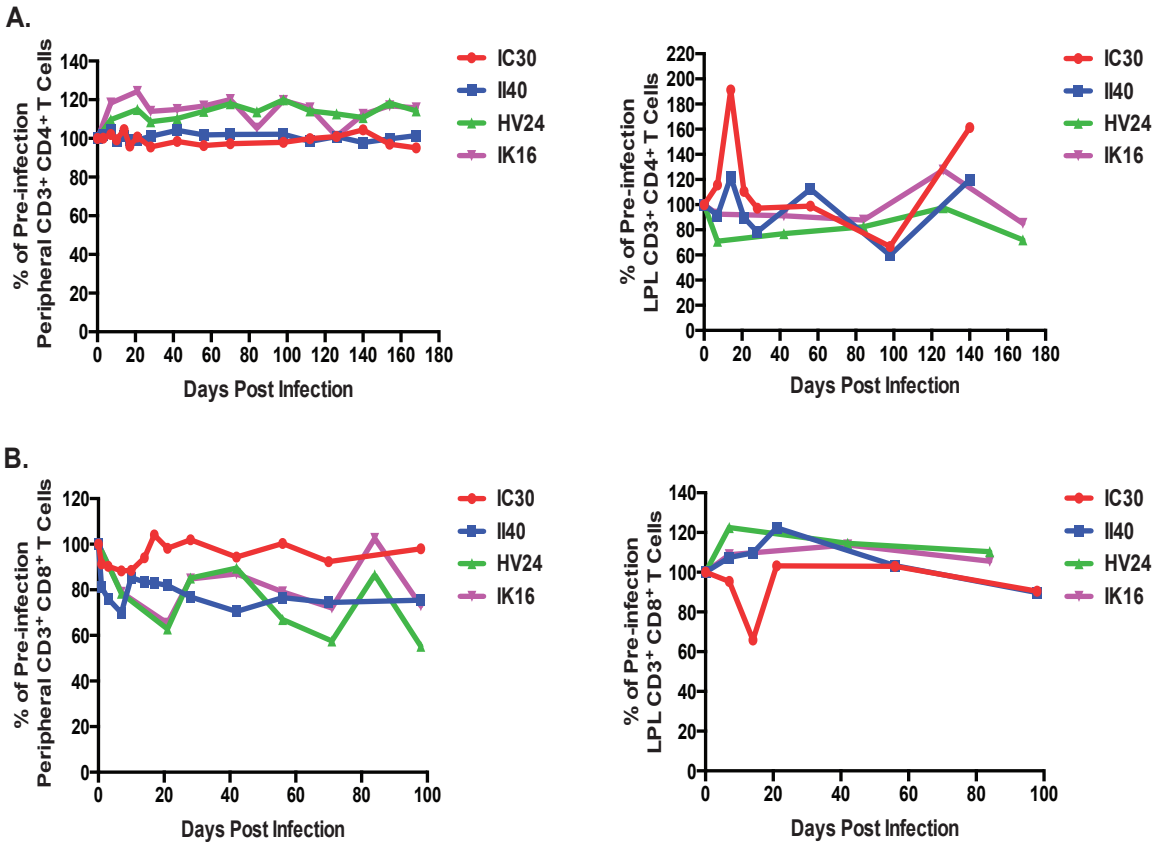


Figure 3-3: CD4+ and CD8+ T cell populations in iMac239-ΔD385 infected rhesus macaques. (A) Percentage of pre-infection CD4+ T cells in blood (Left panel) and in lamina propria (Right panel). **(B)** Percentage of pre-infection CD8+ T cells in blood (Left panel) and in lamina propria (Right panel).

Our previous work had shown that iMac239-ΔD385 was capable of infecting $CD8^+$ T cells in vitro (Chapter 2), thus we hypothesized that $CD8^+$ T cells could also be infected in vivo. In fact, we observed no significant loss of $CD8^+$ T cells in the periphery or lamina propria throughout the first 100 days of infection (Fig. 3-3B).

Taken together, these findings indicate that iMac239-ΔD385 is either not infecting a significant proportion of $CD4^+$ or $CD8^+$ T cells, or if it is, is not causing the death of these cell types. It also suggests that these two key components of cellular immunity are available to contribute to host control of viral replication.

Cellular tropism and anatomic distribution of iMac239-ΔD385 infection

The CD4-independent phenotype of iMac239-ΔD385, and our previous finding of an expanded cell tropism in vitro, raised the possibility of infection of alternative (non-$CD4^+$) cells in vivo. We performed confocal microscopy on tissues from infected animals at peak infection to gain a better understanding of which cell types were infected during acute iMac239-ΔD385 infection. Using colabeling for p27 Gag expression and various cellular markers, we observed infection of $CD3^+$ T cells, $CD68^+$ macrophages, and an as yet unidentified cell type that is CD3-, CD2-, CD68-, CD163-, LN5-, DR-, CD123-, CD209-, CD20-. All three infected cell types were seen in the lymph node and lamina propria at days 7 and 14 post infection (Fig. 3-4A and B).

In addition to an expanded cellular tropism in vivo, we also observed an alteration in the anatomic distribution of infected cells within the lymph node during the acute phase. In contrast to SIVmac239 infection, in which infection in the lymph node is concentrated in the T cell rich cortical region of the lymph node around germinal centers

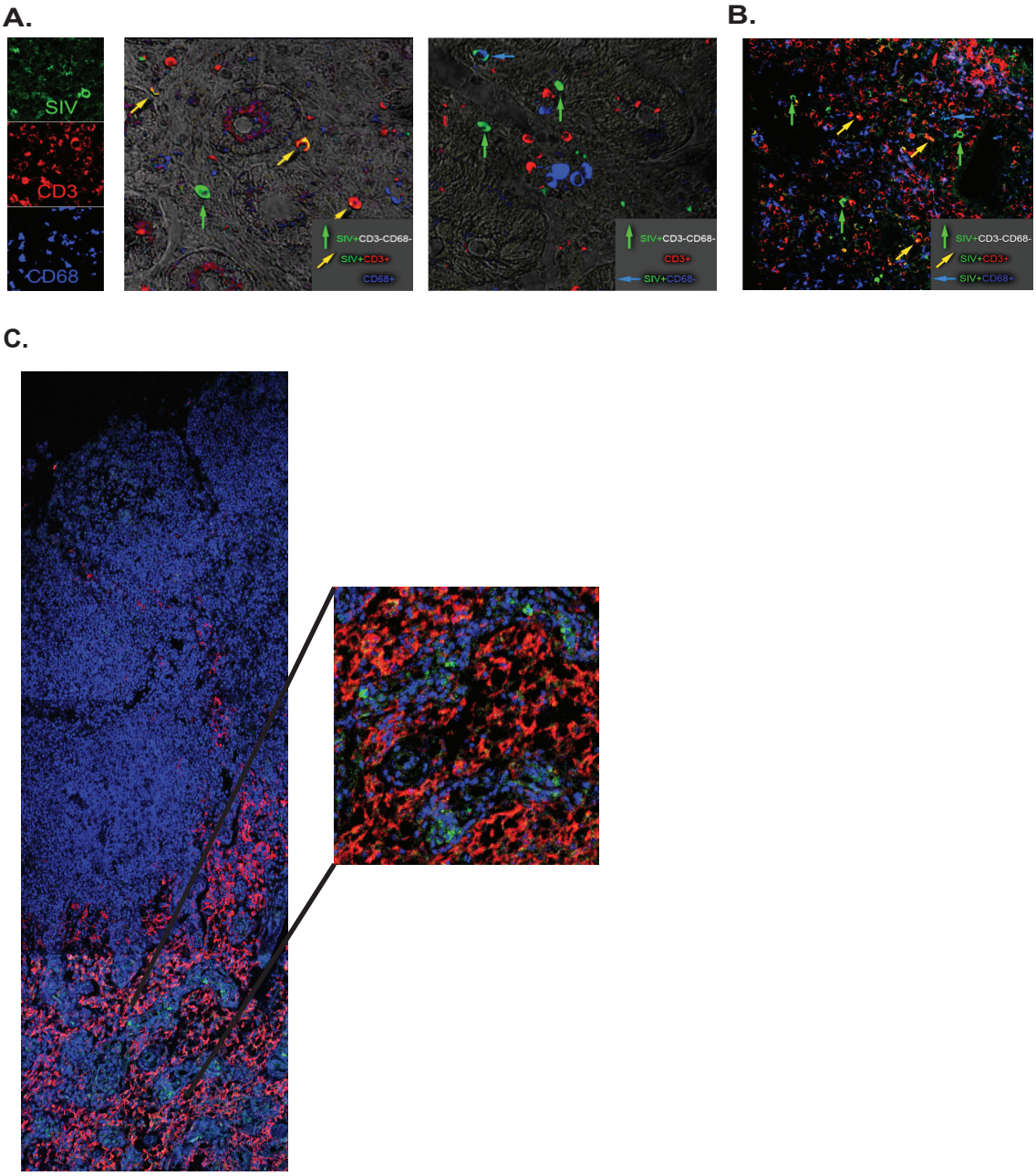


Figure 3-4: Tropism and distribution of iMac239-ΔD385 infection in tissues. Staining of lamina propria at day 7 **(A)** and lymph node at day 14 **(B)** from one representative animal. SIV p27 Gag+ cells are stained in green, CD3+ T cells are stained in red, and CD68+ macrophages are shown in blue. p27 Gag+ CD3+ cells are denoted by yellow arrows, p27 Gag+ CD68+ cells are denoted by blue arrows, and p27 Gag+, CD3-, CD68- cells are denoted by green arrows. **(C)** Staining of a lymph node from one representative animal at peak infection. Blue indicates DAPI staining for nuclear DNA, red indicates macrophage and dendritic cells in the medulla of the lymph node, green staining indicates p27 Gag+ cells. Inset shows a higher magnification of p27 Gag+ cells in the medulla of the lymph node.

(31, 80), tissues from iMac239-ΔD385 infected animals showed the vast majority of infected cells present in the medulla of the lymph node (Fig. 3-4C). These data show a clear diversification in the type of cell targeted and the location of replication during iMac239-ΔD385 in vivo infection.

Depletion of CD8$^+$ T cells to assess their contribution to viral control

To determine the mechanism of viral control, an α-CD8 mAb was administered to animals IC30 and HV24 to deplete peripheral CD8$^+$ T cells at 300 dpi (68). Both animals experienced a total loss of peripheral CD8$^+$ T cells for 30 to 50 days (Fig. 3-5A). Concurrent with the loss of CD8$^+$ T cells, both animals exhibited a low and transient viral peak on the order of 10^2 vRNA copies/mL (Fig. 3-5). Plasma viral replication was once again controlled to undetectable levels upon the reemergence of CD8$^+$ T cells in the periphery. These data are in contrast to other models of control where depletion of CD8$^+$ T cells results in rebound viremia that replicates at levels similar in magnitude to the acute peak (79, 81–84). Based on these findings we can not rule out the CD8$^+$ T cell response as a mechanism of viral control in this model, however it would appear that another mechanism, likely the humoral response, is a major factor contributing to host control of the virus (discussed in further detail below).

Functionality of CD8$^+$ and CD4$^+$ T cells

Based on our observation that CD4$^+$ and CD8$^+$ T cell populations were preserved in all four animals, we tested the functionality of both cell types in the blood and lamina propria at 86 weeks (IC30 and II40) or 37 weeks (HV24 and IK16) post infection. As

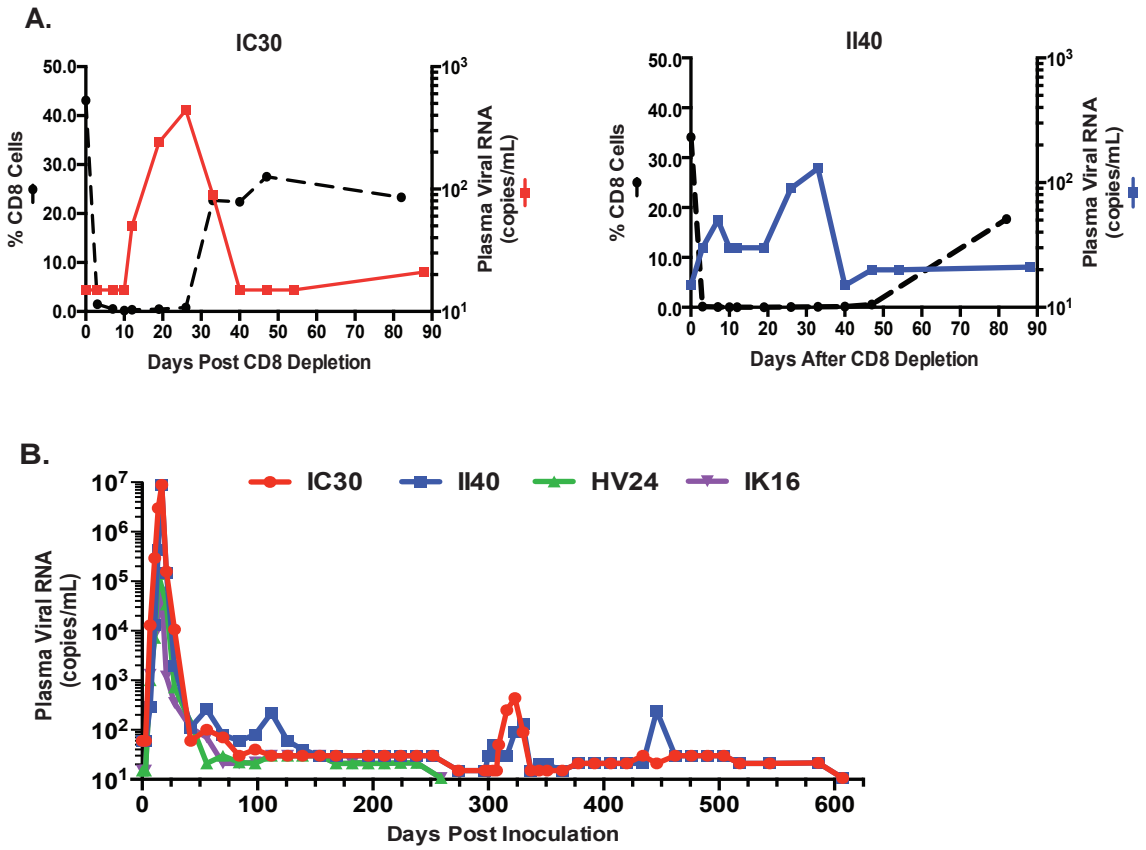


Figure 3-5: CD8+ T cell depletion in two iMac239-ΔD385 infected rhesus macaques. (A) Percentage of peripheral CD8+ T cells and plasma viral loads in animals IC30 (Left panel) and II40 (Right panel) during the CD8+ T cell depletion. **(B)** Plasma viral loads for animals IC30 and II40 over 600dpi, including the CD8+ T cell depletion are shown.

markers of functionality we assessed T cell populations' ability to express the cytokines CD107α, IFNγ, IL-2, IL-17, and TNFα (22, 85, 86). All animals exhibited polyfunctional $CD8^+$ T cell responses in both PBMCs and LPL, although to varying degrees (Fig. 3-6A). Polyfunctional $CD4^+$ T cells were also observed in all four animals in both PBMC and LPL (Fig. 3-6B). Interestingly, of the five cytokines assayed, the degranulation cytokine CD107α (85) was most commonly expressed among $CD8^+$ and $CD4^+$ T cells. The presence of both polyfunctional $CD8^+$ and $CD4^+$ T cells is notable since GALT $CD4^+$ T cells are typically depleted during pathogenic infection (7, 8).

Neutralizing antibody responses

Throughout the course of iMac239-ΔD385 infection we monitored the humoral response by periodically testing the ability of sera to neutralize autologous, homologous, and heterologous strains of SIV. We have previously shown that in contrast to parental SIVmac239, iMac239-ΔD385 is highly sensitive to neutralization by sera from SIV-infected macaques and to anti-SIV Env monoclonal antibodies (Chapter 2). We found that the autologous (iMac239) neutralizing antibody response appeared rapidly, coinciding with the decline in plasma viral load (Fig. 3-7A). This response peaked at an average ID_{50} on the order of 1:100,000 starting at day 42 then declined slightly and was maintained at an average ID_{50} on the order of 1:10,000 throughout the chronic phase, even in the absence of detectable plasma viral load.

The kinetics of the homologous (SIVmac251.6) and heterologous (SIVsmE660/BR-CG7G-IR1) neutralizing antibody responses were similar to that of the autologous response, but the magnitude was approximately one log lower (Fig. 3-7B and

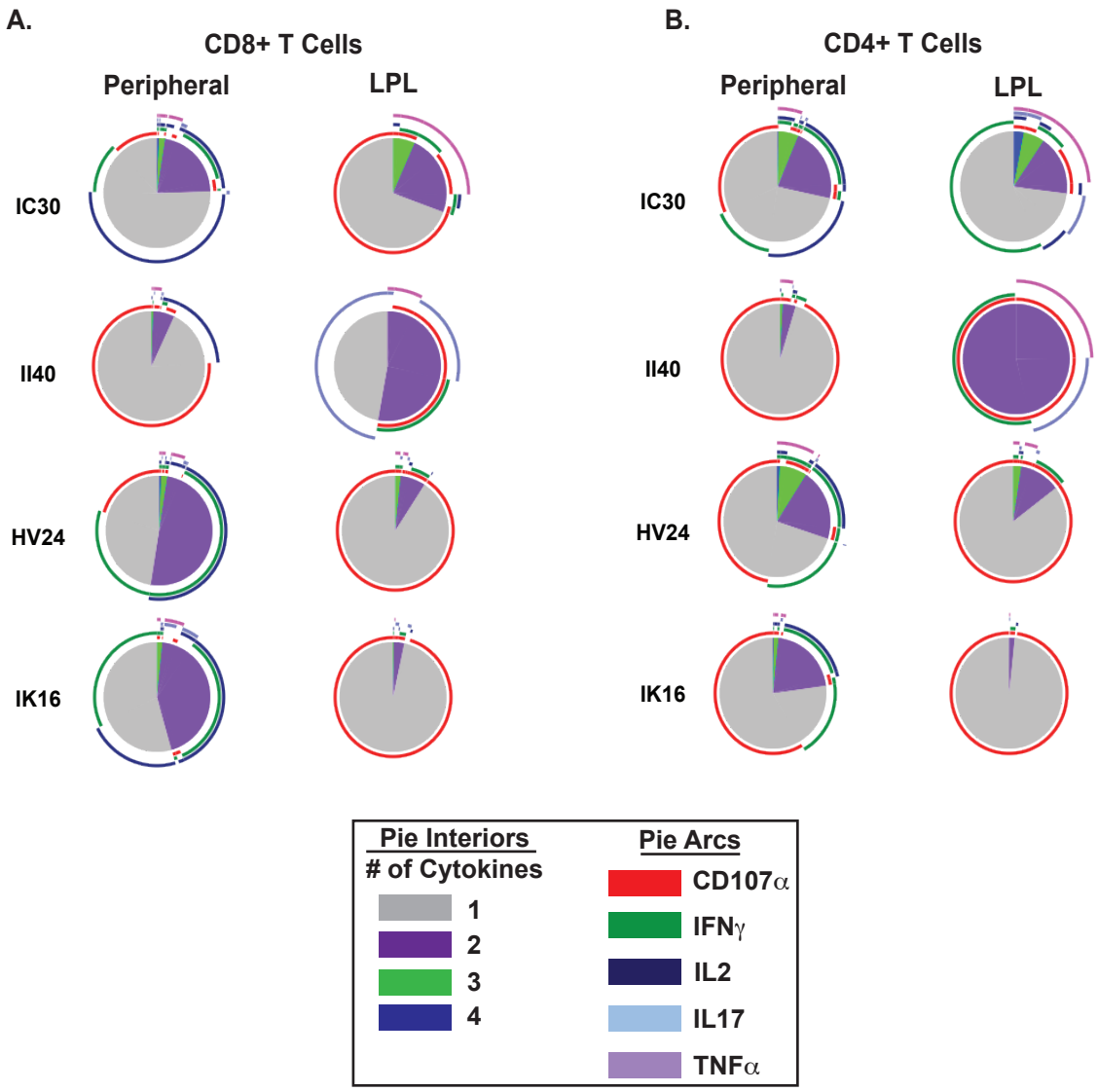


Figure 3-6: CD8+ and CD4+ T cell responses in iMac239-ΔD385 infected rhesus macaques. Polyfunctional responses were quantified for CD8+ **(A)** and CD4+ **(B)** T cells in the periphery and lamina propria. The interior of each pie chart shows the number of cytokines expressed. Pie arcs denote the cytokine expressed.

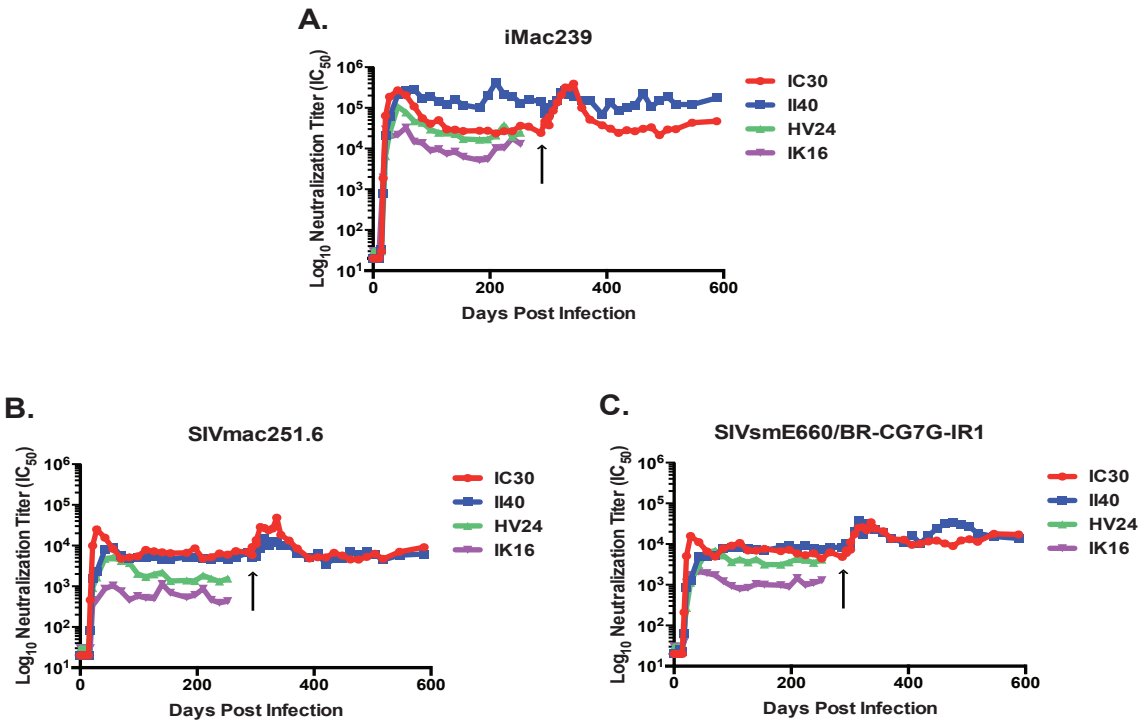


Figure 3-7: Neutralization activity in serum from iMac239-ΔD385 infected rhesus macaques. Neutralization of iMac239 **(A)**, SIVmac251.6 **(B)**, and SIVsmE660/BR-CG7G-IR1 **(C)** pseudotype viruses by sera from all four animals over the course of infection. Arrow indicates the time of CD8+ T cell depletion and transient reemergence of virus resulting in a concurrent boost of antibody responses.

C). In the two animals that underwent a $CD8^+$ T cell depletion at 300 dpi, IC30 and II40, the antibody responses against all strains tested were transiently boosted with the reemergence of virus (Fig. 3-7, indicated by arrows). All three strains tested, iMac239, SIVmac251.6, and SIVsmE660/BR-CG7G-IR1 are considered Tier 1, neutralization sensitive strains (72, 73). Neutralization of more resistant, Tier 2 SIV strains was not observed, nor was any neutralization seen for the highly resistant parental SIVmac239 (data not shown).

Taken together, these data show that iMac239-ΔD385 infection resulted in a high and sustained neutralizing antibody response to Tier 1 strains, which was maintained even in the absence of detectable plasma viral load. This finding would suggest that viral replication is occurring in the tissues and thereby continually priming the antibody response, or that a robust memory response developed during the acute phase of infection. The latter possibility is particularly intriguing given the rarity of iMac239-ΔD385 infected cells in and around lymph node germinal centers during acute infection, and the probable sparing of Tfh cells, which contribute to the generation of long lived plasma B cells (reviewed in (20)).

Viral replication in lymph node

After observing that plasma viral loads were undetectable for over 200 days in all four animals we wondered whether virus was still replicating in tissues, if it was completely latent, or if it had been cleared by the host immune response. To assess the viral load in tissues we amplified viral DNA and RNA from lymph node mononuclear cells at 86 weeks (IC30 and II40) or 37 weeks (HV24 and IK16) post infection. We found

extremely low levels of both viral DNA and RNA at each time point, on the order of one copy of DNA or RNA per 100,000 cells (Table 3-1). This amount of viral RNA in lymph node is approximately 1,000 times less than what has been reported for SIVmac239Δnef, the first live attenuated SIV (87). These results indicate that iMac239-ΔD385 has not in fact been cleared by the host immune response, but is replicating at extremely low levels in the lymph node during the chronic phase of infection.

Preliminary assessment of iMac239-ΔD385 as a live attenuated vaccine

Given the magnitude and longevity of the neutralizing antibody response, in conjunction with both polyfunctional $CD4^+$ and $CD8^+$ T cell responses, we hypothesized that iMac239-ΔD385 may serve as a live attenuated vaccine and protect against a pathogenic challenge. We chose the SIVsmE660 swarm for a challenge stock because it would serve as a heterologous challenge and contains a mixture of neutralization sensitive and neutralization resistant strains (73). The four iMac239-ΔD385 infected animals were genotyped for their Trim5α alleles since these can have an impact on the acquisition of SIVsmE660 as well as viral replication (69, 88–90). Animals IC30 and HV24 were TFP/TFP, corresponding to the most restrictive phenotype; II40 was TFP/Q, the intermediate phenotype; and IK16 was Q/Q the permissive phenotype (Table 3-2). Four naïve rhesus macaques that were also negative for MHC I controlling alleles were used as controls for the challenge experiment. In an attempt to control for the variation in Trim5α alleles of the iMac239-ΔD385 group, naïve control animals with a mix of Trim5α alleles were chosen with one animal carrying TFP/TFP, two animals carrying TFP/Q, and one animal carrying Q/Q (Table 3-2).

Table 3-1: Viral genetic material in lymph nodes of iMac239-ΔD385 animals

	Copies/100,000 Cells	
	DNA	**RNA**
IC30[a]	2.4	1.2
II40[a]	1.6	0.9
HV24[b]	2.5	0.5
IK16[b]	3.0	1.2

[a] 86 weeks post infection
[b] 37 weeks post infection

Table 3-2: Trim5α genotypes of study animals

Group	Animal	Trim5α Genotype	Effect of Trim5α Genotype on Infection
iMac239-ΔD385 Animals	IC30	TFP/TFP	Restrictive
	II40	TFP/Q	Intermediate
	HV24	TFP/TFP	Restrictive
	IK16	Q/Q	Permissive
Control Animals	JN82	TFP/TFP	Restrictive
	JN28	TFP/Q	Intermediate
	JB66	TFP/Q	Intermediate
	JL28	Q/Q	Permissive

All animals were inoculated intrarectally weekly with SIVsmE660 (5,000 $TCID_{50}$). After five weekly challenges, animals with detectable SIVsmE660 plasma virus were excluded from subsequent challenges. Challenge inoculations were stopped after ten weeks. Of the control animals, one animal became infected on the first challenge and the remaining three were infected on the second challenge. Of iMac239-ΔD385 animals, two animals were infected on the second challenge (II40 and IK16), one animal was infected on the seventh challenge (HV24), and one animal remained uninfected after all ten challenges (IC30) (Fig. 3-8A).

The acute peak plasma SIVsmE660 viral load in control animals ranged from 10^6-10^7 RNA copies/mL (Fig. 3-8B and C). After the acute viral peak, two control animals maintained a viral set point approximately one log lower than the acute peak and were carriers of restrictive and permissive Trim5α alleles. The two control animals carrying the TFP/Q alleles controlled their plasma SIVsmE600 viral load to $\leq 10^3$ vRNA copies/mL by 50 dpi (Fig. 3-8C, Left panel).

Of the three iMac239-ΔD385 infected animals that acquired SIVsmE660 post challenge, the acute plasma SIVsmE660 viral load ranged from 10^5-10^6 vRNA copies/mL (Fig. 3-8B). Strikingly, when stratified by Trim5α genotype, a clear one log reduction in peak plasma viral load was observed in iMac239-ΔD385 compared to control animals, however there are not enough animals in this study to determine significance (Fig. 3-8B). One iMac239-ΔD385 infected animal, IK16, maintained a high SIVsmE660 viral load throughout the chronic phase. The two other SIVsmE660-infected iMac239-ΔD385 animals, II40 and HV24, exhibited a slower time to acute viral peak and variable plasma SIVsmE660 viral loads that had multiple chronic phase peaks followed by periods of

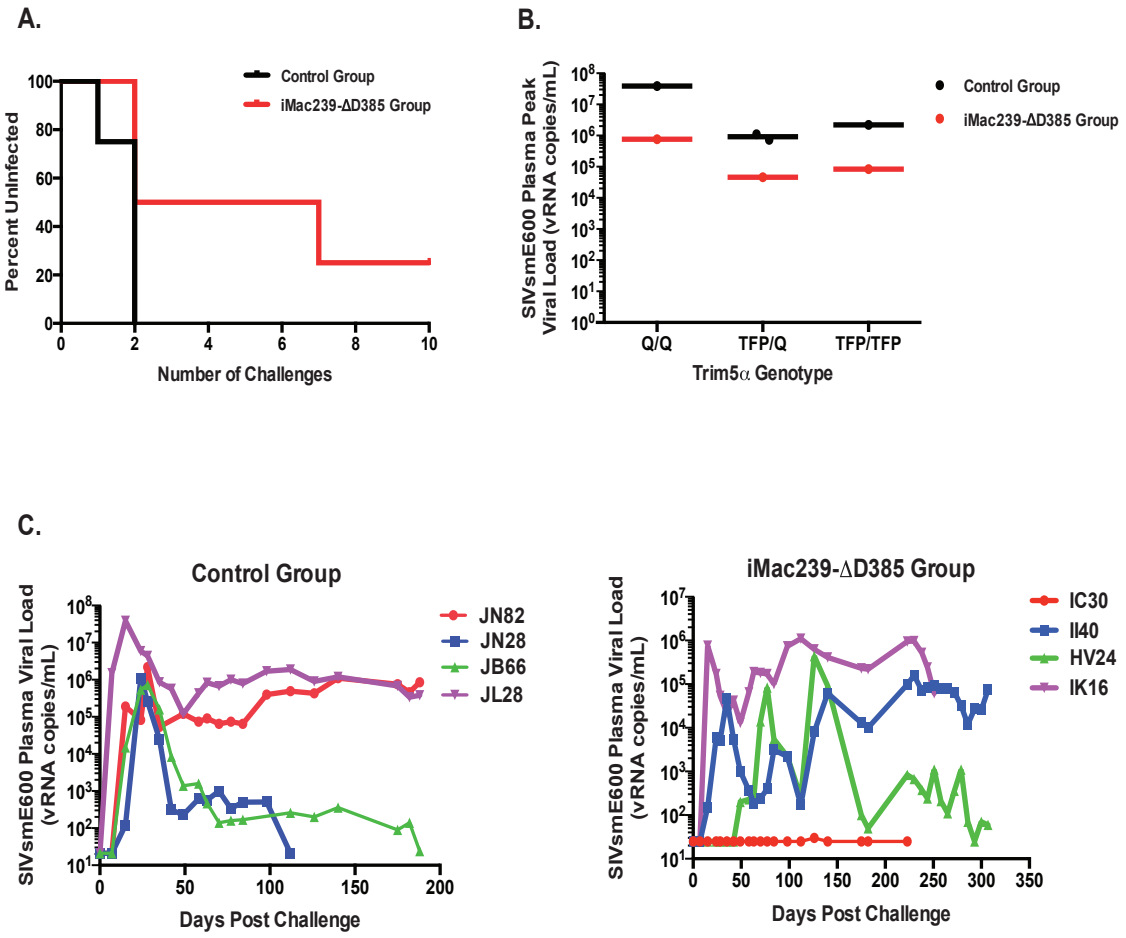


Figure 3-8: Outcome of SIVsmE660 pathogenic challenge. (A) Kaplan-Meir curve indicating the number of inoculations required before infection in the control and iMac239-ΔD385 groups. **(B)** Acute plasma peak viral load for the control group and iMac239-ΔD385 group organized by Trim5α genotype. **(C)** Plasma SIVsmE660 viral loads for the control group (Left panel) and iMac239-ΔD385 group (Right panel) animals.

declining replication. After 200 days the SIVsmE660 viral load had plateaued, a high set-point and a low set-point in II40 and HV24, respectively (Fig. 3-8C, Right panel).

While the number of animals in this experiment is too small to calculate significance, it appears that iMac239-ΔD385 infection may have worked synergistically with the effect of Trim5α alleles to prevent infection in one animal, reduce the acute SIVsmE660 viral load by approximately a log in three animals, and prolong the time to peak SIVsmE660 viral load in two animals.

$CD4^+$ T cell population dynamics post-challenge

As an indicator of disease progression post challenge we monitored the population of $CD4^+$ T cells in SIVsmE660 infected animals. We observed an average 60% drop in the LPL $CD4^+$ T cells in the four control animals by day 28, corresponding with acute viral replication (Fig. 3-9, Left panel). None of the animals were able to recover significant numbers of LPL $CD4^+$ T cells during the chronic phase. Only one iMac239-ΔD385 animal, IK16, lost the vast majority of its LPL $CD4^+$ T cells post challenge (89.2% at 28 dpi). The other two SIVsmE660 infected iMac239-ΔD385 animals ultimately lost 20-40% of their LPL $CD4^+$ T cells, however this loss was gradual and only manifested during the chronic phase of infection (Fig. 3-9, Right panel). Although not statistically significant, these findings suggest that iMac239-ΔD385 infection prior to challenge resulted in an immune response capable of blunting the loss of target LPL $CD4^+$ T cells from SIVsmE660 infection in two of three animals.

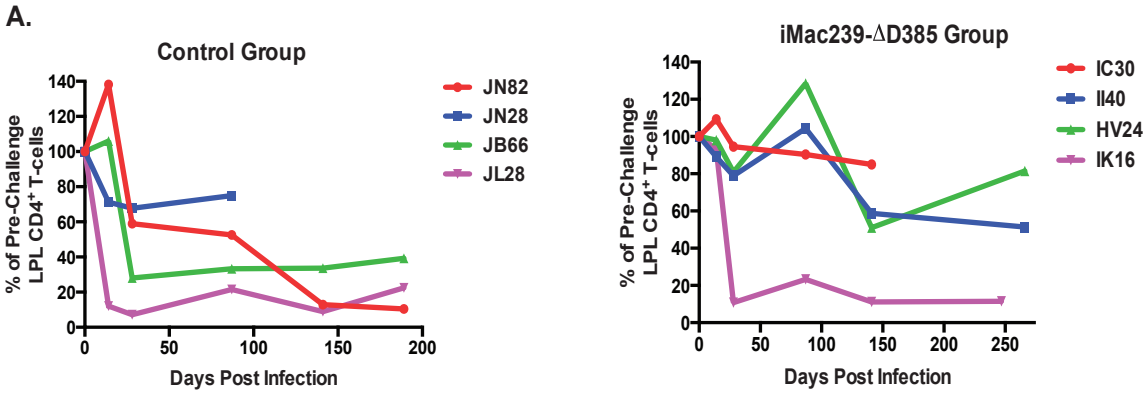


Figure 3-9: CD4+ T cell populations in SIVsmE660 challenged rhesus macaques. Percentage of pre-infection CD4+ T cells in lamina propria in control (Left panel) and iMac239-ΔD385 (Right panel) groups.

Analysis of transmitted founder SIVsmE660 variants

While iMac239-ΔD385 vaccination was not sufficient to prevent the acquisition of SIVsmE660 in three of four animals, we sought to determine whether the antibody response observed in these animals pre-challenge had been able to reduce the number of SIVsmE660 transmitted founder viruses and, or, select for neutralization resistant transmitted founder strains. Utilizing single genome amplification and sequencing of plasma virus at the acute peak and during the chronic phase we were able to determine that two of three iMac239-ΔD385 animals had only one transmitted founder SIVsmE660 strain and the third animal, IK16, had more than ten transmitted founder SIVsmE660 strains (Fig. 3-10, Table 3-3). The four control animals were infected with varying numbers of SIVsmE660 strains either one, two, or six (Table 3-3).

Based on genetic signatures within Env we were able to predict the neutralization phenotype of these transmitted founder (T/F) and chronic phase SIVsmE660 strains in the iMac239-ΔD385 animals (75, 91). There was no clear selection for a neutralization phenotype of transmitted founder variants, the T/F of II40 was neutralization resistant, the T/F of HV24 was neutralization sensitive, and IK16 had a mixed population of variants (Table 3-3). However, during the chronic phase, all three animals selected for a dominant variant that is predicted to be neutralization resistant; this selection occurred within 2-3 months. The predicted neutralization phenotypes of the SIVsmE660 strains in the control animals were not determined.

This study is too small to determine a significant difference in the number of transmitted founder strains between the control and iMac239-ΔD385 groups. While there was no obvious selection for a particular neutralization phenotype in the transmitted

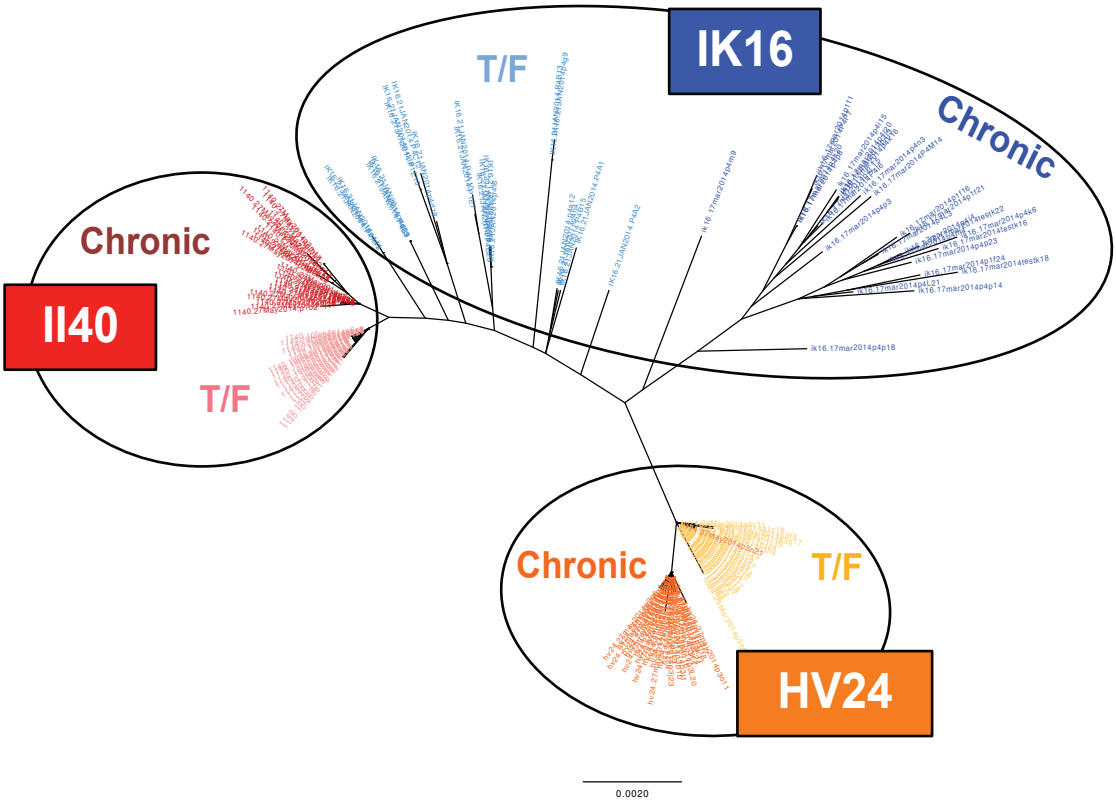


Figure 3-10: Evolution of SIVsmE660 strains in iMac-ΔD385 infected animals. Bayesian phylogeny of the Env amino acid sequences of transmitted founder (T/F) and chronic strains of SIVsmE660 derived from the plasma of SIVsm660 infected iMac239-ΔD385 rhesus macaques by SGA analysis.

Table 3-3: Table of transmitted founder SIVsmE660 strains.

Group	Animal	# of Transmitted Founders	Genetic Prediction of Neutralization Phenotype of Transmitted Founder	Genetic Prediction of Neutralization Phenotype Dominant Variant During Chronic Phase
iMac239-ΔD385 Animals	**II40**	1	Resistant	Resistant
	HV24	1	Sensitive	Resistant
	IK16	≥10	Mixed	Resistant
Control Animals	**JB66**	2	Not Determined	
	JN82	2		
	JN28	1		
	JL28	6		

founder strains of iMac239-ΔD385-infected animals, the presence of a neutralization resistant strain as the dominant chronic phase variant based on genetic signatures in three of three iMac239-ΔD385 animals implies a humoral response that is exerting immune pressure on the viral swarm in these animals.

Antibody responses post challenge

Although infection with iMac239-ΔD385 resulted in strong immune responses, it was only able to protect against SIVsmE660 acquisition in one of four animals. Therefore we were interested in monitoring how the immune responses in iMac239-ΔD385 infected animals might further develop after exposure to an additional SIV. Both control and iMac239-ΔD385 animals rapidly developed high neutralizing titers (~10^6 ID_{50}) to a Tier 1 SIVsmE660 strain, SIVsmE660.2A5-VTRN (Fig. 3-11A, Left panel) (75). All animals developed a much lower neutralizing response (~10^3 ID_{50}) against a Tier 2 neutralization resistant strain, SIVsmE660.2A5-IAKN (75), however this response took a longer time to mature (Fig. 3-11A, Right panel).

We continued to monitor the humoral response over time to determine if the breadth or magnitude of the response would increase. We found two animals, IK16 and II40, whose sera ultimately could neutralize 100% of the Tier 2 neutralization resistant SIVsmE660 strain SIVsmE660.2A5-IAKN, which has not previously been reported (75, 91). The ability to neutralize 100% of this strain did not occur until 16 weeks post infection in IK16 and 45 weeks post infection in II40 (Fig. 3-11B).

While there is no evidence to suggest that the antibody responses in control or iMac239-ΔD385 animals was able to control SIVsmE660 replication, the expansion of

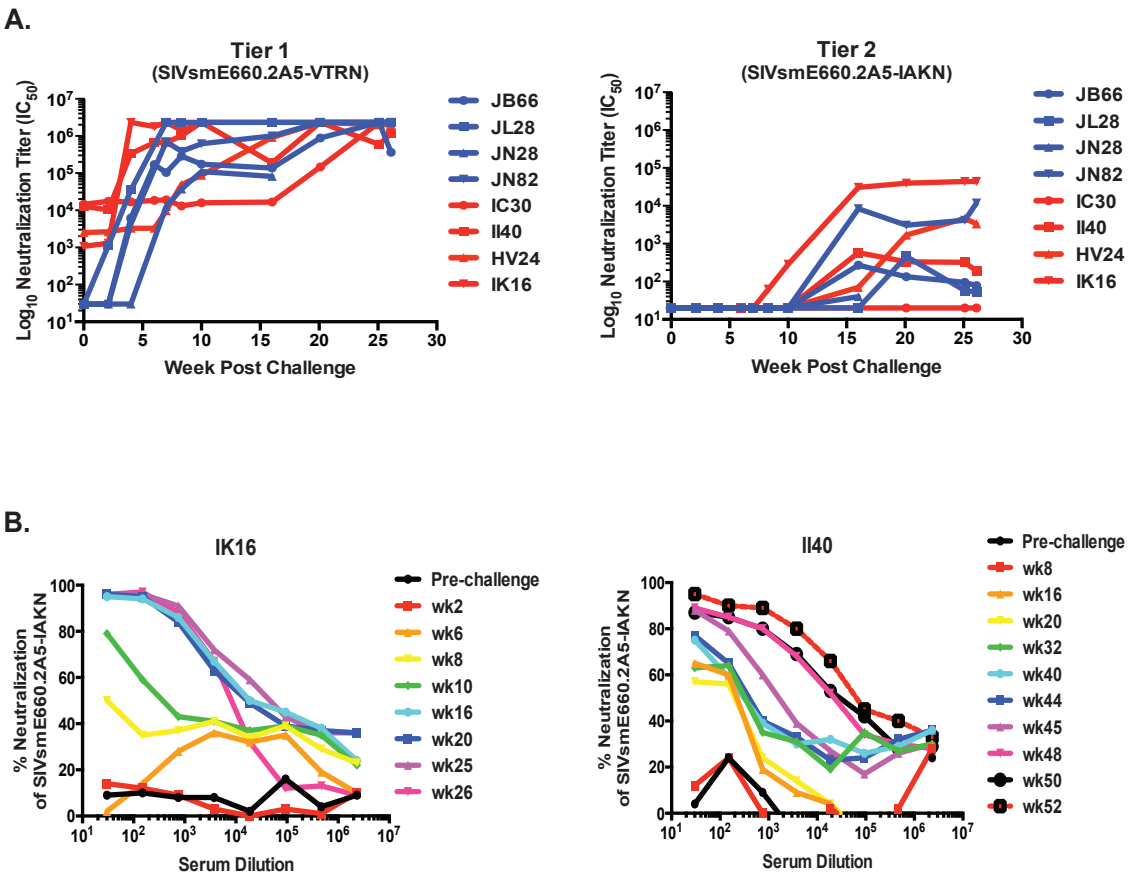


Figure 3-11: Neutralization activity in serum from control and iMac239-ΔD385 animals post SIVsmE660 challenge. (A) Neutralization of a Tier 1 (Left panel) or Tier 2 (Right panel) pseudotype strain of SIVsmE660 over time. **(B)** Percent neutralization of the Tier 2 SIVsmE660.2A5-IAKN pseudotype over time by sera from iMac239-ΔD385 animals IK16 (Left panel) and II40 (Right panel).

breadth and increase in magnitude in two of three iMac239-ΔD385 animals post SIVsmE660 challenge suggests that iMac239-ΔD385 infection may have primed these animals for an improved humoral response. Further work is being done to characterize the epitopes targeted by this neutralizing response.

Discussion

We propose that our novel strain, iMac239-ΔD385, which no longer binds CD4, is the first model of true CD4-independent SIV infection in vivo. As discussed previously, other reported CD4-independent strains of SIV maintain a functional CD4 binding site and presumably will still utilize CD4 for entry in vivo (40, 47, 48). These reported CD4i strains commonly result in low viral loads, macrophage infection, and pathologies associated with macrophage infection, including giant cell pneumonias and neurological disease (41, 48, 56, 55). In contrast, iMac239-ΔD385 infection results in a high acute viremia but without any AIDS related pathologies, despite early macrophage infection. In spite of the ablation of, or significant reduction in, CD4 binding, iMac239-ΔD385 did not appear to suffer a significant fitness cost in vivo with an acute peak similar to that of SIVmac239, albeit at a slight delay (Fig. 3-1). Additionally, in all four animals the virus maintained all of the Env mutations present in the inoculum suggesting that CD4 binding was not required for robust replication and that mutations imparting CD4-independence did not impair replication. One animal showed the acquisition of an additional mutation in the cytoplasmic tail of Env, however it is likely that this mutation is the result of MHC-II mediated immune pressure rather than an attempt to overcome a fitness deficit (Fig. 3-2).

Similar to other live attenuated SIV strains, iMac239-ΔD385 viral replication rapidly declined after the acute peak. Interestingly, in comparison to the live attenuated viruses (LAVs) studied by Fukazawa et al (87), iMac239-ΔD385 had a peak similar to that of SIVmac239Δnef, which had the highest peak of the study group, but iMac239-ΔD385 had a much lower chronic phase set point of undetectable viral replication, most similar to the single cycle SIVmac239 (scSIVmac239). The undetectable plasma viral load in the chronic phase of iMac239-ΔD385 infection suggested that the host immune response had effectively controlled viral replication.

We had hypothesized that a non CD4-tropic virus would no longer selectively target $CD4^+$ T cells in vivo, and that these cells would be preserved during infection. In four of four iMac239-ΔD385 infected animals we observed no significant loss of $CD4^+$ T cells in the periphery or lamina propria, indicating that we had in fact disrupted CD4 tropism in vivo (Fig. 3-3). Our previous work had shown that iMac239-ΔD385 could infect $CD8^+$ $CCR5^+$ T cell in vitro, thus we also investigated whether $CD8^+$ T cells were lost during in vivo infection. We found that, similar to the $CD4^+$ T cell population, $CD8^+$ T cells were maintained at roughly pre-infection levels through the first 100 days of infection (Fig. 3-3). From these results we can draw two conclusions, the first is that both of these cell populations were maintained during iMac239-ΔD385 infection and are available to perform their adaptive immune response functions. Second, the high viral replication during the acute phase, but preservation of these two cell types, suggests that they either are not the primary cell targets or, that infection does not cause widespread cellular death.

Given the apparent conservation of T cells in the periphery and GALT, we used confocal microscopy to determine the immunophenotype of infected cells in the lamina propria and lymph node during peak infection. We observed an expansion in the cell types infected beyond $CD4^+$ T cells with iMac239-ΔD385 infection of $CD3^+$ T cells, $CD68^+$ macrophages, and an as yet unidentified cell type that is negative for the markers: CD3-, CD2-, CD68-, CD163-, LN5-, DR-, CD123-, CD209-, CD20- at peak infection in both the lamina propria and lymph node (Fig. 3-5A and B). Besides the expanded cellular tropism in vivo, we also observed an alteration in the anatomic distribution of infection within the lymph node at peak infection. In particular, very few infected cells were observed in the T cell rich cortical region of the lymph node including in and around germinal centers. Rather, infected cells were predominantly found in the medulla of the lymph node, but were not identified as macrophages or dendritic cells based on confocal microscopy staining (Fig. 3-5C).

The paucity of iMac239-ΔD385 infected cells in the cortex of the lymph node is in sharp contrast to the extensive infection of Tfh cells in this region during pathogenic infection (92). Recent work has highlighted how infection of this cell type contributes to pathogenesis not just through deregulation of Tfh mediated B cell help, perturbations of B cell differentiation, and dysregulated antibody production, but also as a source of the viral reservoir (25–30, 87). Thus the lack of infected Tfh cells in the iMac239-ΔD385 model raises the possibility that these cells will be able to function normally, potentially resulting in an improved humoral response. While we were able to assess the humoral response in iMac239-ΔD385 infected animals, as discussed below, this model requires further investigation to establish the anatomic location and cell type responsible for

harboring the viral reservoir, as well as the dynamics and functionality of the Tfh population.

The infection of macrophages is not surprising given the CD4-independent phenotype of iMac239-ΔD385. The observation of infected macrophages as early as seven days post infection in the GALT and lymph node precedes the appearance of macrophage infection in SIVmac239 infected animals by approximately two weeks (54). We had considered the possibility that iMac239-ΔD385, as a result of efficient macrophage infection, would cause a variety of pathologies associated with macrophage tropic strains, including neurological and pulmonary disease (41, 55, 56). While we observed low levels of viral RNA in the cerebrospinal fluid (CSF) of iMac239-ΔD385-infected animals during the acute phase, the viral load declined to undetectable levels during the chronic phase, mirroring the plasma viral load (data not shown). The lack of detectable viremia in the CSF does not preclude the presence of iMac239-ΔD385 in the brain during the chronic phase, however more extensive sampling of brain tissues is required to determine the extent of infection in this compartment. It is worth noting that none of the animals manifested outward clinical signs of brain or pulmonary disease, suggesting that damage due to infection or inflammation in these tissues was minimal.

The evidence of multiple cell targets and the prevalence of these cells ($CD3^+$ T cells and macrophages in particular), suggested that the undetectable plasma viral load during the chronic phase of infection was not due to lack of cellular targets but rather the result of immune control. To understand the mechanism of viral control and determine if virus had been cleared, two animals underwent a $CD8^+$ T cell depletion 300 days post infection. The $CD8^+$ T cell depletion resulted in a low (~10^2 vRNA copies/mL) and

transient viral peak, with plasma viral loads once again becoming undetectable upon reappearance of peripheral $CD8^+$ T cells approximately 40 days after the initial depletion (Fig. 3-5A). In these two animals the plasma viral load remained undetectable for another 300 days (Fig. 3-5B) at which point we assessed the amount of virus in the lymph node to determine if virus was still replicating in tissues. In all four animals (two at 86 wpi, two at 37 wpi, Table 3-1) we observed approximately one copy of RNA and one copy of DNA per 100,000 cells, the lowest reported for other live attenuated SIVs (87). Thus, while virus had not been cleared, it was under exquisite host control.

Although the reemerging plasma viral peak during the $CD8^+$ T cell depletion was low, suggesting a non- $CD8^+$ T cell mechanism of viral control, we assessed the functional responses of $CD8^+$ and $CD4^+$ T cells in the periphery and lamina propria. We found that all four animals produced polyfunctional T cell responses in both compartments (Fig. 3-6); however further work is required to determine if these cells are in fact capable of killing infected cells. Interestingly, the most commonly expressed cytokine in both $CD8^+$ and $CD4^+$ T cells was CD107α, whose expression has been correlated with viral control of SIV after vaccination (93) and in elite controllers of HIV-1 (94, 95). This result implies that viral control may be possible through a T cell mediated mechanism, either through $CD4^+$ (96) or $CD8^+$ (97) cytotoxic responses, but under the current conditions of the model other aspects of the immune system obscure the effect of this response.

In all four animals, a high neutralizing antibody response emerged against autologous, homologous, and heterologous Tier 1 strains during the acute phase and coincided with the decline in plasma viral load (Fig. 3-7). Strikingly, this neutralizing

response was maintained at high levels during chronic infection when plasma viral load was undetectable and viral replication in tissues was extremely low. These data, coupled with retargeting of viral infection away from germinal centers, suggest a robust humoral memory response, rather than continual antigen stimulation of the immune system is the source of high neutralizing antibody titers throughout chronic infection.

Based on our observations of polyfunctional $CD8^+$ and $CD4^+$ T cell responses in conjunction with a high and sustained neutralizing antibody response, we tested whether iMac239-ΔD385 could serve as an effective live attenuated vaccine against a pathogenic challenge. The four iMac239-ΔD385 animals, along with Trim5α matched naïve control animals were challenged intrarectally with a SIVsmE660 swarm once a week for up to ten weeks. All four control animals and three of four iMac239-ΔD385 animals became infected with SIVsmE660. Follow-up studies with larger groups of study animals are required to determine if there is a significant reduction in acquisition of SIVsmE660 after iMac239-ΔD385 infection. When separated by Trim5α genotype, we observed an approximate one log decrease in acute SIVsmE660 plasma viral load in iMac239-ΔD385 animals as compared to control animals, however the number of animals in this study is too small to determine significance (Fig. 3-8). Interestingly, while the prototypic loss of GALT $CD4^+$ T cells was observed in control animals after SIVsmE660 infection, depletion of this magnitude only occurred in one iMac239-ΔD385 animal (Fig. 3-9). This finding implies that the immune responses induced by iMac239-ΔD385 infection were sufficient for continued protection of the $CD4^+$ T compartment in two of three animals, even in the face of high SIVsmE660 viral loads.

To determine if iMac239-ΔD385 vaccination was partially effective as measured by a reduction of transmitted founder strains or a sieving of the challenge inoculum such that only neutralization resistant SIVsmE660 strains were able to cross the mucosa, we assessed the number of transmitted founder and chronic strains and performed sequence analysis to predict their neutralization phenotype based on genetic signatures within Env (Fig. 3-10, Table 3)(75). We observed a trend towards a reduction in transmitted founder strains in the iMac239-ΔD385 animals and selection for predicted neutralization resistant strains during the chronic phase; the predicted neutralization phenotype variants in the control group animals remains to be determined. Given the apparent selection of a predicted neutralization resistant SIVsmE660 in iMac239-ΔD385 animals, we assessed the anti-SIVsmE660 neutralizing antibody response in both groups. We found that while initially unable to neutralize a Tier 2 neutralization resistant SIVsmE660 strain, two of three SIVsmE660 infected iMac239-ΔD385 animals ultimately developed a humoral response that was able to neutralize 100% of this variant (Fig. 3-11). This finding is particularly striking because it is the first report of 100% neutralization of this particular Tier 2 strain (75).

This novel in vivo model showcases not only the role of CD4 binding for SIV, but also highlights the consequences of CD4 tropism for the host immune system. In the context of a primate lentivirus that no longer specifically targets $CD4^+$ T cells, the adaptive immune system is preserved and able to mount both cellular and humoral functional responses. It has long been appreciated that the loss of $CD4^+$ T cells during pathogenic HIV and SIV infections results in the collapse of the adaptive immune system, increasing the host's susceptibility to opportunistic infections, thus it perhaps is

not surprising that in a model where $CD4^+$ T cells are preserved the adaptive immune system remains functional.

It is well understood that CD4-independent viruses have Env trimers with a more "open" conformation (98, 99), as if CD4 was already bound; however this is achieved through genetic mutation rather than ligand binding. We have shown that SIV does not require CD4 tropism for robust replication in vitro and in vivo, however one consequence of this trimer formation is a significant increase in neutralization sensitivity (Chapter 2)(39, 100–102). Thus, while the virus is capable of high viral replication, in vivo it would be highly susceptible to the host humoral response, a plausible reason why such variants rarely arise in vivo (39, 103, 104). This susceptibly, coupled with an intact adaptive immune response is the most likely explanation for the extremely low levels for viral replication throughout the chronic phase.

A common phenomenon in live attenuated vaccine models is the inverse relationship between the degree of attenuation of the vaccine strain and efficacy (105, 106). The results from two animals within our model recapitulated this effect, by which the magnitude of the initial iMac239-ΔD385 viral load correlated with the magnitude of both cellular and humoral responses, and ultimately, in conjunction with Trim5α alleles, resulted in drastically different outcomes post-challenge. Specifically, animal IC30 had the highest acute iMac239-ΔD385 viral load, the highest neutralizing antibody response, the greatest proportion of polyfunctional T cells, and the restrictive Trim5α genotype, while animal IK16 had the lowest levels of these three parameters and the permissive Trim5α genotype. IC30 was fully protected against ten SIVsmE660 intrarectal challenges, in contrast to IK16, which was infected on the second challenge and exhibited

the highest level of SIVsmE660 replication and greatest loss of $CD4^+$ T cells of all iMac239-ΔD385 animals. Although the inverse relationship between attenuation and efficacy coupled with the possibility of reversion to pathogenicity through mutation have precluded the development of live attenuated vaccines for HIV clinical trials, they remain one of the two most successful SIV vaccination strategies to date (reviewed in (107, 108))(109), and thus are valuable models for studying correlates of protection.

The development of an improved humoral response, both in expansion of breadth and increase in magnitude, in two of three iMac239-ΔD385 animals that became infected with SIVsmE660 is tantalizing for the implication that iMac239-ΔD385 infection may have served as a prime for the humoral immune system. While it is clear that this improved antibody response was unable to dampen viral replication for the host in which it arose, it is nonetheless intriguing. Current work is focused on isolating novel antibodies capable of neutralizing 100% of the Tier 2 SIVsmE660-2A5.IAKN strain from iMac239-ΔD385 animal IK16. Such an antibody would be a valuable reagent, not only for inclusion in neutralizing antibody panels used for characterizing novel strains of SIV, but also for the structural and binding data that could inform the antibody's mechanism of neutralization. Elucidating this mechanism will be important for understanding the neutralization "lawn chair" effect seen with SIVsmE660-2A5.IAKN, in which maximal inhibition of infection by the molecular clone occurs at only 50% (75, 91)

Future studies exploring the role of a CD4-sparing prime in improving the quality of the humoral response could include adding a CD4-independent Env to a sequential vaccination series. In such a system, exposing the host to an initial prime of an open conformation CD4-independent Env (iMac239-ΔD385), followed by a boost of a closed

conformation CD4-dependent Env (SIVsmE660), through a series of soluble Env trimers (110, 111) may result in a similarly broad humoral response and recapitulate our model.

In summary, our novel model provides insight into the relationship between the viral Env and CD4 and its effect on the immune system. Taken together, these results suggest that viral engagement of CD4 is not required for robust replication, but rather contributes to pathogenesis by allowing for multiple conformations to shield vulnerable epitopes and by targeting a critical component of the adaptive immune system.

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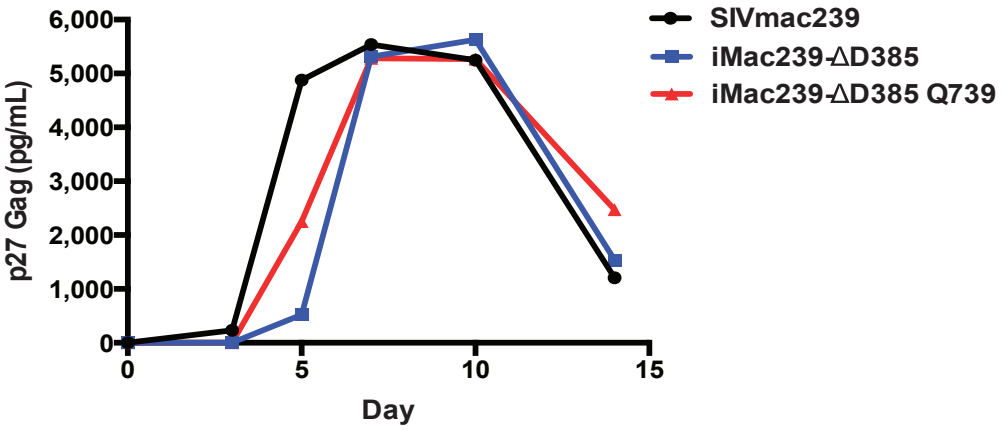

Supplemental Figure 3-1: Replication of Q739 mutant in rhesus PBMCs. Replication of SIVmac239, iMac239-ΔD385, and iMac239-ΔD385 Q739 in ConA/IL-2 stimulated rhesus PBMCs is shown. p27 Gag in culture supernatants was quantified by ELISA at the indicated time points. Results from a representative experiment are shown.

References

1. **Daniel MD**, **Letvin NL**, **King NW**, **Kannagi M**, **Sehgal PK**, **Hunt RD**, **Essex M**, **Desrosiers RC**. 1985. Isolation of T-Cell Tropic HTLV-III-Like Retrovirus from Macaques. Science **228**:1201–1204.

2. **Kestler H**, **Kodama T**, **Ringler D**, **Marthas M**, **Pedersen N**, **Lackner AA**, **Regier D**, **Sehgal P**, **Daniel M**, **King N**. 1990. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science **248**:1109–12.

3. **Chen Z**, **Gettie A**, **Ho DD**, **Marx PA**. 1998. Primary SIVsm isolates use the CCR5 coreceptor from sooty mangabeys naturally infected in west Africa: a comparison of coreceptor usage of primary SIVsm, HIV-2, and SIVmac. Virology **246**:113–124.

4. **Chen Z**, **Zhou P**, **Ho DD**, **Landau NR**, **Marx PA**. 1997. Genetically divergent strains of simian immunodeficiency virus use CCR5 as a coreceptor for entry. J Virol **71**:2705–2714.

5. **Kirchhoff F**, **Pöhlmann S**, **Hamacher M**, **Means RE**, **Kraus T**, **Uberla K**, **Di Marzio P**. 1997. Simian immunodeficiency virus variants with differential T-cell and macrophage tropism use CCR5 and an unidentified cofactor expressed in CEMx174 cells for efficient entry. J Virol **71**:6509–16.

6. **Smith SM**, **Holland B**, **Russo C**, **Dailey PJ**, **Marx PA, Connor RI**. 1999. Retrospective analysis of viral load and SIV antibody responses in rhesus macaques infected with pathogenic SIV: predictive value for disease progression. AIDS Res Hum Retroviruses **15**:1691–1701.

7. **Veazey RS**, **DeMaria M**, **Chalifoux L V**, **Shvetz DE**, **Pauley DR**, **Knight HL**, **Rosenzweig M**, **Johnson RP**, **Desrosiers RC**, **Lackner AA**. 1998. Gastrointestinal Tract as a Major Site of CD4+ T Cell Depletion and Viral Replication in SIV Infection. Science **280**:427–431.

8. **Veazey RS**, **Mansfield KG**, **Tham IC**, **Carville AC**, **Shvetz DE**, **Forand AE**, **Lackner AA**. 2000. Dynamics of CCR5 expression by CD4(+) T cells in lymphoid tissues during simian immunodeficiency virus infection. J Virol **74**:11001–7.

9. **Miller CJ**, **Genescà M**, **Abel K**, **Montefiori D**, **Forthal D**, **Bost K**, **Li J**, **Favre D**, **McCune JM**. 2007. Antiviral antibodies are necessary for control of simian immunodeficiency virus replication. J Virol **81**:5024–35.

10. **Schmitz JE**, **Kuroda MJ**, **Santra S**, **Simon MA**, **Lifton MA**, **Lin W**, **Piatak M**, **Lifson JD**, **Gelman RS**, **Racz P**, **Mansfield KA**, **Letvin NL**, **David C**, **Reimann KA**, **Schmitz E**, **Khunkhun R**, **Grosschupff G**, **Tenner-racz K**, **Montefiori DC**. 2003. Effect of Humoral Immune Responses on Controlling Viremia during Primary Infection of Rhesus Monkeys with Simian Immunodeficiency Virus Effect of Humoral Immune Responses on Controlling Viremia during Primary Infection of Rhesus Monkeys with Simian Immuno. J Virol **77**:2165–2173.

11. **Sodora DL**, **Silvestri G**. 2008. Immune activation and AIDS pathogenesis. AIDS **22**:439–446.

12. **Mothé BR**, **Weinfurter J**, **Wang C**, **Rehrauer W**, **Wilson N**, **Allen TM**, **Allison DB**, **Watkins DI**. 2003. Expression of the Major Histocompatibility Complex Class I Molecule Mamu-A * 01 is associated with control of Simian Immunodeficiency Virus SIV mac 239 replication. J Virol **77**:2736.

13. **Yant LJ**, **Friedrich TC**, **Johnson RC**, **May GE**, **Maness NJ**, **Enz AM**, **Lifson JD**, **Connor DHO**, **Carrington M**, **Watkins DI**. 2006. The High-Frequency Major Histocompatibility Complex Class I Allele Mamu-B * 17 Is Associated with Control of Simian Immunodeficiency Virus SIVmac239 Replication. J Virol **80**:5074–5077.

14. **Loffredo JT**, **Maxwell J**, **Qi Y**, **Glidden CE**, **Borchardt GJ**, **Soma T**, **Bean AT**, **Beal DR**, **Wilson NA**, **Rehrauer WM**, **Lifson JD**, **Carrington M**, **Watkins DI**. 2007. Mamu-B*08-Positive Macaques Control Simian Immunodeficiency Virus Replication. J Virol **81**:8827–8832.

15. **Baskin GB**, **Murphey-Corb M**, **Watson EA**, **Martin LN**. 1988. Necropsy findings in rhesus monkeys experimentally infected with cultured simian immunodeficiency virus (SIV)/delta. Vet Pathol **25**:456–467.

16. **McClure HM**, **Anderson DC**, **Fultz PN**, **Ansari AA**, **Lockwood E**, **Brodie A**. 1989. Spectrum of Disease in Macaque Monkeys Chronically Infected with SIV / SMM. Vet Immunol Immunopathol **21**:13–24.

17. **Zhu J**, **Paul WE**. 2010. Peripheral CD4 T cell differentiation regulated by networks of cytokines and transcription factors. Immunol Rev **238**:247–262.

18. **Yamane H**, **Paul WE**. 2013. Early signaling events that underlie fate decisions of naive CD4+ T cells towards distinct T-helper cell subsets. Immunol Rev **252**:12–23.

19. **Ekkens MJ**, **Shedlock DJ**, **Jung E**, **Troy A**, **Pearce EL**, **Shen H**, **Pearce EJ**. 2007. Th1 and Th2 Cells Help CD8 T-Cell Responses. Infect Immun **75**:2291–2296.

20. **Crotty S**. 2015. A brief history of T cell help to B cells. Nat Rev Immunol **15**:185–9.

21. **Bour-jordan H**, **Bluestone JA**. 2009. Regulating the regulators: costimulatory signals control the homeostatsis and function of regulatory T cells. Immunol Rev **229**:41–66.

22. **Klatt NR**, **Brenchley JM**. 2011. Th17 Cell Dynamics in HIV Infection. Curr Opin HIV AIDS **5**:135–140.

23. **Estes JD**, **Harris LD**, **Klatt NR**, **Tabb B**, **Pittaluga S**, **Paiardini M**, **Barclay GR**, **Smedley J**, **Pung R**, **Oliveira KM**, **Hirsch VM**, **Silvestri G**, **Douek DC**, **Miller CJ**, **Haase AT**, **Lifson J**, **Brenchley JM**. 2010. Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections. PLoS Pathog **6**(8): e1001052. doi:10.1371/journal.ppat.1001052.

24. **Brenchley JM**. 2013. Mucosal immunity in human and simian immunodeficiency lentivirus infections. Mucosal Immunol **6**:657–65.

25. **Cubas RA**, **Mudd JC**, **Savoye A**, **Perreau M**, **Van J**, **Metcalf T**, **Connick E**, **Meditz A**, **Freeman GJ**, **Jr GA**, **Jacobson JM**, **Brooks AD**, **Crotty S**, **Estes JD**, **Pantaleo G**, **Lederman MM**, **Haddad EK**. 2013. Inadequate T follicular cell help impairs B cell immunity during HIV infection. Nat Med **19**(4) doi:10.1038/nm.3109.

26. **Perreau M**, **Savoye A-L**, **De Crignis E**, **Corpataux J-M**, **Cubas R**, **Haddad EK**, **De Leval L**, **Graziosi C**, **Pantaleo G**. 2013. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. J Exp Med **210**:143–156.

27. **Boswell KL**, **Paris R**, **Boritz E**, **Ambrozak D**, **Yamamoto T**, **Darko S**, **Wloka K**, **Wheatley A**, **Narpala S**, **McDermott A**, **Roederer M**, **Haubrich R**, **Connors M**, **Ake J**, **Douek DC**, **Kim J**, **Petrovas C**, **Koup RA.** 2014. Loss of Circulating CD4 T Cells with B Cell Helper Function during Chronic HIV Infection. PLoS Pathog **10**:1–14.

28. **Vinuesa CG**. 2012. HIV and T follicular helper cells: a dangerous relationship. J Clin Invest **122**:3059–62.

29. **Lindqvist M**, **Lunzen J Van**, **Soghoian DZ**, **Kuhl BD**, **Ranasinghe S**, **Kranias G**, **Flanders MD**, **Cutler S**, **Yudanin N**, **Muller MI**, **Davis I**, **Farber D**, **Hartjen P**, **Haag F**, **Alter G**, **Schulze J**, **Streeck H**. 2012. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. J Clin Invest **122**:3271–3280.

30. **Onabajo OO**, **Mattapallil JJ**. 2013. Expansion or Depletion of T Follicular Helper cells During HIV Infection: Consequences for B cell Responses. Curr HIV Res **11**:595–600.

31. **Fukazawa Y**, **Lum R**, **Okoye AA**, **Park H**, **Matsuda K**, **Bae JY**, **Hagen SI**, **Shoemaker R**, **Deleage C**, **Lucero C**, **Morcock D**, **Swanson T**, **Legasse AW**, **Axthelm MK**, **Hesselgesser J**, **Geleziunas R**, **Hirsch VM**, **Edlefsen PT**, **Piatak M**, **Estes JD**, **Lifson JD**, **Picker LJ**. 2015. B cell follicle sanctuary permits persistent productive simian immunodeficiency virus infection in elite controllers. Nat Med **21**:132–139.

32. **Chun TW**, **Finzi D**, **Margolick J**, **Chadwick K**, **Schwartz D**, **Siliciano RF**. 1995. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. Nat Med **1**:1284–1290.

33. **Finzi D**, **Hermankova M**, **Pierson T**, **Carruth LM**, **Buck C**, **Chaisson RE**, **Quinn TC**, **Chadwick K**, **Margolick J**, **Brookmeyer R**, **Gallant J**, **Markowitz M**, **Ho DD**, **Richman DD**, **Siliciano RF**. 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science **278**:1295–1300.

34. **Finzi D**, **Blankson J**, **Siliciano JD**, **Margolick JB**, **Chadwick K**, **Pierson T**, **Smith K**, **Lisziewicz J**, **Lori F**, **Flexner C**, **Quinn TC**, **Chaisson RE**, **Rosenberg E**, **Walker B**, **Gange S**, **Gallant J**, **Siliciano RF**. 1999. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat Med **5**:512–517.

35. **Chirmule N**, **Kalyanaraman VS**, **Oyaizu N**, **Slade HB**, **Pahwa S**. 1990. Inhibition of functional properties of tetanus antigen-specific T-cell clones by envelope glycoprotein GP120 of human immunodeficiency virus. Blood **75**:152–159.

36. **Cefai D**, **Debre P**, **Kaczorek M**, **Autran B**, **Bismuth G**. 1990. Human Immunodeficiency Virus-1 glycoproteins gpl2O and gp160 specifically inhibit the CD3 / T cell-antigen receptor phosphoinositide transduction pathway. J Clin Invest **86**:2117–2124.

37. **Schwartz O**, **Alizon M**, **Heard J-M**, **Danos L**. 1994. Impairment of T cell receptor-dependent stimulation in CD4+ lymphocytes after contact with membrane-bound HIV-1 envelope glycoprotein. Virology **198**:360–365.

38. **Banapour B**, **Marthas ML**, **Ramos RA**, **Lohman BL**, **Unger RE**, **Gardner MB**, **Pedersen NC**, **Luciw PA**. 1991. Identification of viral determinants of macrophage tropism for simian immunodeficiency virus SIVmac. J Virol **65**:5798–5805.

39. **Puffer BA**, **Pöhlmann S**, **Edinger AL**, **Carlin D**, **Sanchez MD**, **Reitter J**, **Watry DD**, **Fox HS**, **Desrosiers RC**, **Doms RW**. 2002. CD4 Independence of Simian Immunodeficiency Virus Envs Is Associated with Macrophage Tropism , Neutralization Sensitivity , and Attenuated Pathogenicity. J Virol **76**:2595–2605.

40. **Puffer BA**, **Altamura LA**, **Pierson TC**, **Doms RW**. 2004. Determinants within gp120 and gp41 contribute to CD4 independence of SIV Envs. Virology **327**:16–25.

41. **Ortiz AM**, **Klatt NR**, **Li B**, **Yi Y**, **Tabb B**, **Hao XP**, **Sternberg L**, **Lawson B**, **Carnathan PM**, **Cramer EM**, **Engram JC**, **Little DM**, **Ryzhova E**, **Gonzalez-Scarano F**, **Paiardini M**, **Ansari AA**, **Ratcliffe S**, **Else JG**, **Brenchley JM**, **Collman RG**, **Estes JD**, **Derdeyn CA**, **Silvestri G**. 2011. Depletion of CD4 + T cells abrogates post-peak decline of viremia in SIV-infected rhesus macaques. J Clin Invest **121**:4433–45.

42. **Yen P-J**, **Herschhorn A**, **Haim H**, **Salas I**, **Gu C**, **Sodroski J**, **Gabuzda D**. 2014. Loss of a conserved N-linked glycosylation site in the simian immunodeficiency virus envelope glycoprotein V2 region enhances macrophage tropism by increasing CD4-independent cell-to-cell transmission. J Virol **88**:5014–28.

43. **Kodama T**, **Wooley DP**, **Naidu YM**, **Kestler HW**, **Daniel MD**, **Li Y**, **Desrosiers RC**. 1989. Significance of Premature Stop Codons in env of Simian Immunodeficiency Virus. J Virol **63**:4709–4714.

44. **Zhu P**, **Chertova E**, **Bess J**, **Lifson JD**, **Arthur LO**, **Liu J**, **Taylor KA**, **Roux KH**. 2003. Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions. Proc Natl Acad Sci **100**:15812–15817.

45. **Johnston PB**, **Dubay JW**, **Hunter E**. 1993. Truncations of the simian immunodeficiency virus transmembrane protein confer expanded virus host range by removing a block to virus entry into cells. J Virol **67**:3077–3086.

46. **Bonavia, A, Bullock, BT, Gisselman, KM, Margulies, BJ, Clements, JE**. 2005. A single amino acid change and truncated TM are sufficient for simian immunodeficiency virus to enter cells using CCR5 in a CD4-independent pathway. Virology **341**:12–23.

47. **Flaherty MT**, **Hauer DA**, **Mankowski JL**, **Zink MC**, **Clements JE**. 1997. Molecular and biological characterization of a neurovirulent molecular clone of simian immunodeficiency virus. J Virol **71**:5790–8.

48. **Luciw PA**, **Shaw KE**, **Unger RE**, **Planelles V**, **Stout MW**, **Lackner JE**, **Pratt-Lowe E**, **Leung NJ**, **Banapour B**, **Marthas ML**. 1992. Genetic and biological comparisons of pathogenic and nonpathogenic molecular clones of simian immunodeficiency virus (SIVmac). AIDS Res Hum Retroviruses **8**:395–402.

49. **Gallo SA**, **Puri A**, **Blumenthal R**. 2001. HIV-1 gp41 six-helix bundle formation occurs rapidly after the engagement of gp120 by CXCR4 in the HIV-1 Env-mediated fusion process. Biochemistry **40**:12231–6.

50. **Daniel MD**, **Kirchhoff F**, **Czajak SC**, **Sehgal PK**, **Desrosiers RC**. 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science **258**:1938–1941.

51. **Gibbs JS**, **Lackner AA**, **Lang SM**, **Simon MA**, **Sehgal PK**, **Daniel MD**, **Desrosiers RC**. 1995. Progression to AIDS in the absence of a gene for vpr or vpx. J Virol **69**:2378–2383.

52. **Ilyinskii PO**, **Daniel MD**, **Simon MA**, **Lackner AA**, **Desrosiers RC**. 1994. The role of upstream U3 sequences in the pathogenesis of simian immunodeficiency virus-induced AIDS in rhesus monkeys. J Virol **68**:5933–44.

53. **Picker LJ**, **Hagen SI**, **Lum R**, **Reed-Inderbitzin EF**, **Daly LM**, **Sylwester AW**, **Walker JM**, **Siess DC**, **Piatak M**, **Wang C**, **Allison DB**, **Maino VC**, **Lifson JD**, **Kodama T**, **Axthelm MK**. 2004. Insufficient Production and Tissue Delivery of CD4+Memory T Cells in Rapidly Progressive Simian Immunodeficiency Virus Infection. J Exp Med **200**:1299–1314.

54. **Borda JT**, **Alvarez X**, **Kondova I**, **Aye P**, **Simon MA**, **Desrosiers RC**, **Lackner AA**. 2004. Cell tropism of simian immunodeficiency virus in culture is not predictive of in vivo tropism or pathogenesis. Am J Pathol **165**:2111–2122.

55. **Micci L**, **Alvarez X**, **Iriele RI**, **Ortiz AM**, **Ryan ES**, **McGary CS**, **Deleage C**, **McAtee BB**, **He T**, **Apetrei C**, **Easley K**, **Pahwa S**, **Collman RG**, **Derdeyn C a.**, **Davenport MP**, **Estes JD**, **Silvestri G**, **Lackner A a.**, **Paiardini M**. 2014. CD4 Depletion in SIV-Infected Macaques Results in Macrophage and Microglia Infection with Rapid Turnover of Infected Cells. PLoS Pathog **10**(10): e1004467. doi:10.1371/journal.ppat.1004467.

56. **Mankowski JL**, **Flaherty MT**, **Spelman JP**, **Hauer DA**, **Didier PJ**, **Amedee AM**, **Murphey-Corb M**, **Kirstein LM**, **Muñoz A**, **Clements JE**, **Zink MC**. 1997. Pathogenesis of simian immunodeficiency virus encephalitis: viral determinants of neurovirulence. J Virol **71**:6055–60.

57. **Yen P-J**, **Mefford ME**, **Hoxie JA**, **Williams KC**, **Desrosiers RC**, **Gabuzda D**. 2014. Identification and characterization of a macrophage-tropic SIV envelope glycoprotein variant in blood from early infection in SIVmac251-infected macaques. Virology **458-459**:53–68.

58. **Goldstein S**, **Elkins W**, **London W**, **Hahn A**, **Goeken R**, **Martin J**, **Hirsch V**. 1994. Immunization with whole inactivated vaccine protects from infection by SIV grown in human but not macaque cells. J Med Primatol **2-3**:75–82.

59. **Cline AN**, **Bess JW**, **Piatak M**, **Lifson JD**. 2005. Highly sensitive SIV plasma viral load assay: practical considerations, realistic performance expectations, and application to reverse engineering of vaccines for AIDS. J Med Primatol **34**:303–12.

60. **Lifson JD**, **Rossio JL**, **Piatak M**, **Bess J**, **Chertova E**, **Schneider DK**, **Coalter VJ**, **Poore B**, **Kiser RF**, **Imming RJ**, **Scarzello AJ**, **Henderson LE**, **Alvord WG**, **Hirsch VM**, **Benveniste RE**, **Arthur LO**. 2004. Evaluation of the safety, immunogenicity, and protective efficacy of whole inactivated simian immunodeficiency virus (SIV) vaccines with conformationally and functionally intact envelope glycoproteins. AIDS Res Hum Retroviruses **20**:772–87.

61. **Ling B**, **Veazey RS**, **Luckay A**, **Penedo C**, **Xu K**, **Lifson JD**, **Marx PA**. 2002. SIV(mac) pathogenesis in rhesus macaques of Chinese and Indian origin compared with primary HIV infections in humans. AIDS **16**:1489–96.

62. **Wang X**, **Das A**, **Lackner AA**, **Veazey RS**, **Pahar B**. 2008. Intestinal double-positive CD4 ♀ CD8 ♀ T cells of neonatal rhesus macaques are proliferating , activated memory cells and primary targets for SIV MAC251 infection. Blood **112**:4981–4991.

63. **Veazey RS**, **Lifson JD**, **Pandrea I**, **Purcell J**, **Piatak M**, **Lackner AA**. 2003. Simian immunodeficiency virus infection in neonatal macaques. J Virol **77**:8783–92.

64. **Pahar B**, **Cantu MA**, **Zhao W**, **Kuroda MJ**, **Veazey RS**, **Montefiori DC**, **Clements JD**, **Aye PP**, **Lackner AA**, **Lovgren-Bengtsson K**, **Sestak K**. 2006. Single epitope mucosal vaccine delivered via immuno-stimulating complexes induces low level of immunity against simian-HIV. Vaccine **24**:6839–49.

65. **Breed MW**, **Jordan APO**, **Aye PP**, **Sugimoto C**, **Alvarez X**, **Kuroda MJ**, **Pahar B**, **Keele BF**, **Hoxie JA**, **Lackner AA**. 2013. A single amino acid mutation in the envelope cytoplasmic tail restores the ability of an attenuated simian immunodeficiency virus mutant to deplete mucosal CD4+ T cells. J Virol **87**:13048–52.

66. **Pahar B**, **Wang X**, **Dufour J**, **Lackner AA**, **Veazey RS**. 2007. Virus-specific T cell responses in macaques acutely infected with SHIVsf162p3. Virology **363**:36–47.

67. **Faul EJ**, **Aye PP**, **Papaneri AB**, **Pahar B**, **McGettigan JP**, **Schiro F**, **Chervoneva I**, **Montefiori DC**, **Lackner AA**, **Schnell MJ**. 2009. Rabies virus-based vaccines elicit neutralizing antibodies, poly-functional CD8+ T cell, and protect rhesus macaques from AIDS-like disease after SIV(mac251) challenge. Vaccine **28**:299–308.

68. **Schmitz JE**, **Kuroda MJ**, **Santra S**, **Sasseville VG**, **Simon MA**, **Lifton MA**, **Racz P**, **Tenner-Racz K**, **Dalesandro M**, **Scallon BJ**, **Ghrayeb J**, **Forman MA**, **Montefiori DC**, **Rieber EP**, **Letvin NL**, **Reimann KA**. 1999. Control of viremia in Simian Immunodeficiency Virus infection by CD8+ lymphocytes. Science **283**:857–860.

69. **Kirmaier A**, **Wu F**, **Newman RM**, **Hall LR**, **Morgan JS**, **O'Connor S**, **Marx PA**, **Meythaler M**, **Goldstein S**, **Buckler-White A**, **Kaur A**, **Hirsch VM**, **Johnson WE**. 2010. TRIM5 suppresses cross-species transmission of a primate immunodeficiency virus and selects for emergence of resistant variants in the new species. PLoS Biol **8**:35–36.

70. **Montefiori DC**. 2001. Evaluating Neutralizing Antibodies Against HIV, SIV, and SHIV in Luciferase Reporter Gene Assays. Current Protocols in Immunology. John Wiley & Sons, Inc.

71. **Montefiori DC**. 2005. Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. Curr Protoc Immunol **Chapter 12**:Unit 12.11.

72. **Means RE**, **Greenough T**, **Desrosiers RC**. 1997. Neutralization sensitivity of cell culture-passaged simian immunodeficiency virus. J Virol **71**:7895–902.

73. **Lopker M**. 2013. Heterogeneity in neutralization sensitivities of viruses comprising the simian immunodeficiency virus SIVsmE660 isolate and vaccine challenge stock. J Virol **87**:5477–5492.

74. **Keele BF**. 2009. Low-dose rectal inoculation of rhesus macaques by SIVsmE660 or SIVmac251 recapitulates human mucosal infection by HIV-1. J Exp Med **206**:1117–1134.

75. **Roederer M**, **Keele BF**, **Schmidt SD**, **Mason RD**, **Welles HC**, **Fischer W**, **Labranche C**, **Foulds KE**, **Louder MK**, **Yang Z-Y**, **Todd J-PM**, **Buzby AP**, **Mach LV**, **Shen L**, **Seaton KE**, **Ward BM**, **Bailer RT**, **Gottardo R**, **Gu W**, **Ferrari G**, **Alam SM**, **Denny TN**, **Montefiori DC**, **Tomaras GD**, **Korber BT**, **Nason MC**, **Seder RA**, **Koup RA**, **Letvin NL**, **Rao SS**, **Nabel GJ**, **Mascola JR**. 2014. Immunological and Virological Mechanisms of Vaccine-Mediated Protection Against SIV and HIV. Nature **505**:502–508.

76. **Vinet-Oliphant H**, **Alvarez X**, **Buza E**, **Borda JT**, **Mohan M**, **Aye PP**, **Tuluc F**, **Douglas SD**, **Lackner AA.** 2010. Neurokinin-1 Receptor (NK1-R) Expression in the Brains of SIV-Infected Rhesus Macaques. Am J Pathol **177**:1286–1297.

77. **Harris RS**, **Liddament MT**. 2004. Retroviral restriction by APOBEC proteins. Nat Rev Immunol **4**:868–877.

78. **Dzuris JL**, **Sidney J**, **Horton H**, **Correa R**, **Carter D**, **Chesnut RW**, **Watkins DI**, **Sette A**. 2001. Molecular Determinants of Peptide Binding to Two Common Rhesus Macaque Major Histocompatibility Complex Class II Molecules. J Virol **75**:10958–10968.

79. **Breed MW**, **Elser SE**, **Torben W**, **Jordan APO**, **Aye PP**, **Midkiff C**, **Schiro F**, **Sugimoto C**, **Alvarez-Hernandez X**, **Blair RV**, **Somasunderam A**, **Utay NS**, **Kuroda MJ**, **Pahar B**, **Wiseman RW**, **O'Connor DH**, **LaBranche CC**, **Montefiori DC**, **Marsh M**, **Li Y**, **Piatak M**, **Lifson JD**, **Keele BF**, **Fultz PN**, **Lackner AA**, **Hoxie JA.** 2015. Elite control, gut CD4 T cell sparing, and enhanced mucosal T cell responses in *Macaca nemestrina* infected by an SIV lacking a gp41 trafficking motif. J Virol JVI.01134–15.

80. **Lackner AA**, **Vogel P**, **Ramos RA**, **Kluge JD**, **Marthas M**. 1994. Early events in tissues during infection with pathogenic (SIVmac239) and nonpathogenic (SIVmac1A11) molecular clones of simian immunodeficiency virus. Am J Pathol **145**:428–439.

81. **Jin X**, **Bauer DE**, **Tuttleton SE**, **Lewin S**, **Gettie A**, **Blanchard J**, **Irwin CE**, **Safrit JT**, **Mittler J**, **Weinberger L**, **Kostrikis LG**, **Zhang L**, **Perelson AS**, **Ho DD**. 1999. Dramatic rise in plasma viremia after CD8+ T cell depletion in Simian Immunodeficiency Virus-infected macaques. Journal Exp Med **189**:991–998.

82. **Matano T**, **Shibata R**, **Siemon C**, **Connors M**, **Lane HC**, **Martin MA**. 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. J Virol **72**:164–9.

83. **Schmitz JE**, **Kuroda MJ**, **Santra S**, **Sasseville VG**, **Simon MA**, **Lifton MA**, **Racz P**, **Tenner-Racz K**, **Dalesandro M**, **Scallon BJ**, **Ghrayeb J**, **Forman MA**, **Montefiori DC**, **Rieber EP**, **Letvin NL**, **Reimann KA**. 1999. Control of viremia in Simian Immunodeficiency Virus infection by CD8+ lymphocytes. Science **283**:857–860.

84. **Metzner KJ**, **Jin X**, **Lee F V**, **Gettie A**, **Bauer DE**, **Di Mascio M**, **Perelson AS**, **Marx PA**, **Ho DD**, **Kostrikis LG**, **Connor RI**. 2000. Effects of in vivo CD8(+) T cell depletion on virus replication in rhesus macaques immunized with a live, attenuated simian immunodeficiency virus vaccine. J Exp Med **191**:1921–1931.

85. **Betts MR**, **Brenchley JM**, **Price DA**, **De Rosa SC**, **Douek DC**, **Roederer M**, **Koup RA**. 2003. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods **281**:65–78.

86. **Kedzierska K**, **Crowe SM**. 2001. Cytokines and HIV-1: Interactions and clinical implications. Antivir Chem Chemother **12**:133–150.

87. **Fukazawa Y**, **Park H**, **Cameron MJ**, **Lefebvre F**, **Lum R**, **Coombes N**, **Mahyari E**, **Hagen S**, **Bae JY**, **Reyes III MD**, **Swanson T**, **Legasse AW**, **Sylwester A**, **Hansen SG**, **Smith AT**, **Stafova P**, **Shoemaker R**, **Li Y**, **Oswald K**, **Axthelm MK**, **McDermott A**, **Ferrari G**, **Montefiori DC**, **Edlefsen PT**, **Piatak Jr. M**, **Lifson JD**, **Sekaly RP**, **Picker LJ**. 2012. Lymph node T cell responses predict the efficacy of live attenuated SIV vaccines. Nat Med **18**:1673–1681.

88. **Reynolds MR**, **Sacha JB**, **Weiler AM**, **Borchardt GJ**, **Glidden CE**, **Sheppard NC**, **Norante F a**, **Castrovinci P a**, **Harris JJ**, **Robertson HT**, **Friedrich TC**, **McDermott AB**, **Wilson NA**, **Allison DB**, **Koff WC**, **Johnson WE**, **Watkins DI**. 2011. The TRIM5{alpha} Genotype of Rhesus Macaques Affects Acquisition of Simian Immunodeficiency Virus SIVsmE660 Infection after Repeated Limiting-Dose Intrarectal Challenge. J Virol **85**:9637–40.

89. **Yeh WW**, **Rao SS**, **Lim S-Y**, **Zhang J**, **Hraber PT**, **Brassard LM**, **Luedemann C**, **Todd JP**, **Dodson A**, **Shen L**, **Buzby AP**, **Whitney JB**, **Korber BT**, **Nabel GJ**, **Mascola JR**, **Letvin NL**. 2011. The TRIM5 gene modulates penile mucosal acquisition of simian immunodeficiency virus in rhesus monkeys. J Virol **85**:10389–98.

90. **Lim SY**. 2010. TRIM5alpha modulates immunodeficiency virus control in rhesus monkeys. PLoS Pathog **6**:e1000738.

91. **Lee F-H**, **Mason R**, **Welles H**, **Learn GH**, **Keele BF**, **Roederer M**, **Bar KJ**. 2015. Breakthrough virus neutralization resistance as a correlate of protection in a nonhuman primate heterologous SIV vaccine challenge study. J Virol JVI.01531–15.

92. **Lackner AA**, **Vogel P**, **Ramos RA**, **Kluge JD**, **Marthas M**. 1994. Early events in tissues during infection with pathogenic (SIVmac239) and nonpathogenic (SIVmac1A11) molecular clones of simian immunodeficiency virus. Am J Pathol **145**:428–439.

93. **Yamamoto T**, **Johnson MJ**, **Price DA**, **Wolinsky DI**, **Almeida JR**, **Petrovas C**, **Nason M**, **Yeh WW**, **Shen L**, **Roederer M**, **Rao SS**, **McDermott AB**, **Lefebvre F**, **Nabel GJ**, **Haddad EK**, **Letvin NL**, **Douek DC**, **Koup RA**. 2012. Virus Inhibition Activity of Effector Memory CD8+ T Cells Determines Simian Immunodeficiency Virus Load in Vaccinated Monkeys after Vaccine Breakthrough Infection. J Virol **86**:5877–5884.

94. **Hersperger AR**, **Pereyra F**, **Nason M**, **Demers K**, **Sheth P**, **Shin LY**, **Kovacs CM**, **Rodriguez B**, **Sieg SF**, **Teixeira-Johnson L**, **Gudonis D**, **Goepfert PA**, **Lederman MM**, **Frank I**, **Makedonas G**, **Kaul R**, **Walker BD**, **Betts MR**. 2010. Perforin Expression Directly Ex Vivo by HIV-Specific CD8+ T-Cells Is a Correlate of HIV Elite Control. PLoS Pathog **6**:e1000917.

95. **Freel SA**, **Lamoreaux L**, **Chattopadhyay PK**, **Saunders K**, **Zarkowsky D**, **Overman RG**, **Ochsenbauer C**, **Edmonds TG**, **Kappes JC**, **Cunningham CK**, **Denny TN**, **Weinhold KJ**, **Ferrari G**, **Haynes BF**, **Koup RA**, **Graham BS**, **Roederer M**, **Tomaras GD**. 2010. Phenotypic and functional profile of HIV-inhibitory CD8 T cells elicited by natural infection and heterologous prime/boost vaccination. J Virol **84**:4998–5006.

96. **Soghoian, DZ**, **Streeck H**. 2010. Cytolytic CD4+ T cells in viral immunity. Expert Rev Vaccines **9**:1453–1463.

97. **O'Connell KA**, **Bailey JR**, **Blankson JN**. 2009. Elucidating the elite: mechanisms of control in HIV-1 infection. Trends Pharmacol Sci **30**:631–637.

98. **White TA**, **Bartesaghi A**, **Borgnia MJ**, **Meyerson JR**, **de la Cruz MJV**, **Bess JW**, **Nandwani R**, **Hoxie JA**, **Lifson JD**, **Milne JLS**. 2010. Molecular Architectures of Trimeric SIV and HIV-1 Envelope Glycoproteins on Intact Viruses : Strain- Dependent Variation in Quaternary Structure. PLoS Pathog **6**(12): e1001249. doi:10.1371/journal.ppat.1001249.

99. **White TA**, **Bartesaghi A**, **Borgnia MJ**, **de la Cruz MJV**, **Nandwani R**, **Hoxie JA**, **Bess JW**, **Lifson JD**, **Milne JLS**, **Subramaniam S**. 2011. Three-Dimensional Structures of Soluble CD4-Bound States of Trimeric Simian Immunodeficiency Virus Envelope Glycoproteins Determined by Using Cryo-Electron Tomography. J Virol **85**:12114–12123.

100. **Hoffman TL**, **LaBranche CC**, **Zhang W**, **Canziani G**, **Robinson J**, **Chaiken I**, **Hoxie JA**, **Doms RW**. 1999. Stable exposure of the coreceptor-binding site in a CD4-independent HIV-1 envelope protein. PNAS **96**:6359–64.

101. **Edwards TG**, **Hoffman TL**, **Branche CCLA**, **Romano J**, **Adkinson J**, **Sharron M**, **Hoxie JA**, **Doms RW**. 2001. Relationships between CD4 Independence , Neutralization Sensitivity , and Exposure of a CD4-Induced Epitope in a Human Immunodeficiency Virus Type 1 Envelope Protein. J Virol **75**:5230–5239.

102. **Kolchinsky P**, **Kiprilov E**. 2001. Increased Neutralization Sensitivity of CD4-Independent Human Immunodeficiency Virus Variants. J Virol **75**:2041–2050.

103. **Francella N**, **Gwyn SE**, **Yi Y**, **Li B**, **Xiao P**, **Elliott STC**, **Ortiz AM**, **Hoxie JA**, **Paiardini M**, **Silvestri G**, **Derdeyn CA**, **Collman RG**. 2013. CD4+ T Cells Support Production of Simian Immunodeficiency Virus Env Antibodies That Enforce CD4-Dependent Entry and Shape Tropism In Vivo. J Virol **87**:9719–9732.

104. **Francella N**, **Elliott ST**, **Yi Y**, **Gwyn SE**, **Ortiz AM**, **Li B**, **Silvestri G**, **Paiardini M**, **Derdeyn CA**, **Collman RG**. 2013. Decreased plasticity of coreceptor use by CD4-independent SIV Envs that emerge in vivo. Retrovirology **10**:133.

105. **Wyand MS**, **Manson KH**, **Garcia-Moll M**, **Montefiori D**, **Desrosiers RC**. 1996. Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. J Virol **70**:3724–3733.

106. **Johnson RP**, **Lifson JD**, **Czajak SC**, **Cole KS**, **Manson KH**, **Glickman R**, **Yang J**, **Montefiori DC**, **Montelaro R**, **Wyand MS**, **Desrosiers RC**. 1999. Highly attenuated vaccine strains of simian immunodeficiency virus protect against vaginal challenge: inverse relationship of degree of protection with level of attenuation. J Virol **73**:4952–4961.

107. **Lifson JD**, **Haigwood NL**. 2012. Lessons in nonhuman primate models for AIDS vaccine research: from minefields to milestones., p. 463–482. *In* HIV From Biology to Prevention and Treatment.

108. **Koff WC**, **Johnson PR**, **Watkins DI**, **Burton DR**, **Lifson JD**, **Hasenkrug KJ**, **McDermott AB**, **Schultz A**, **Zamb TJ**, **Boyle R**, **Desrosiers RC**. 2006. HIV vaccine design: insights from live attenuated SIV vaccines. Nat Immunol **7**:19–23.

109. **Hansen SG**, **Jr MP**, **Ventura AB**, **Hughes CM**, **Gilbride M**, **Ford JC**, **Oswald K**, **Shoemaker R**, **Li Y**, **Matthew S**, **Gilliam AN**, **Xu G**, **Whizin N**, **Burwitz BJ**, **Planer L**, **Turner JM**, **Legasse AW**, **Axthelm MK**, **Nelson JA**, **Früh K**, **Sacha JB**, **Estes JD**, **Keele BF**, **Edlefsen PT**, **Lifson JD**, **Picker LJ**. 2014. Immune clearance of highly pathogenic SIV infection. Nature **502**:doi:10.1038/nature12519.

110. **Sanders RW**, **Derking R**, **Cupo A**, **Julien J-P**, **Yasmeen A**, **de Val N**, **Kim HJ**, **Blattner C**, **de la Peña AT**, **Korzun J**, **Golabek M**, **de Los Reyes K**, **Ketas TJ**, **van Gils MJ**, **King CR**, **Wilson I a**, **Ward AB**, **Klasse PJ**, **Moore JP**. 2013. A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. PLoS Pathog **9**(9): e1003618. doi:10.1371/journal.ppat.1003618.

111. **Sanders RW**, **van Gils MJ**, **Derking R**, **Sok D**, **Ketas TJ**, **Burger JA**, **Ozorowski G**, **Cupo A**, **Simonich C**, **Goo L**, **Arendt H**, **Kim HJ**, **Lee JH**, **Pugach P**, **Williams M**, **Debnath G**, **Moldt B**, **van Breemen MJ**, **Isik G**, **Medina-Ramirez M**, **Back JW**, **Koff WC**, **Julien J-P**, **Rakasz EG**, **Seaman MS**, **Guttman M**, **Lee KK**, **Klasse PJ**, **LaBranche C**, **Schief WR**, **Wilson IA**, **Overbaugh J**, **Burton DR**, **Ward AB**, **Montefiori DC**, **Dean H**, **Moore JP**. 2015. HIV-1 neutralizing antibodies induced by native-like envelope trimers. Science **349**: doi:10.1126/science.aac4223.

CHAPTER 4

Conclusions and Future Directions

Overview

HIV-1 infection in humans is characterized by the targeting of $CD4^+$ T cells that express at least one of two chemokine coreceptors, CCR5 and CXCR4 (1–3). The depletion of the $CD4^+$ T cell population ultimately leads to the collapse of the adaptive immune system and renders the host susceptible to opportunistic infections (reviewed in (4)). Despite high rates of viral mutation and recombination, usage of CD4 as the primary receptor is an invariant feature (1, 5). For these reasons, usage of CD4 by HIV-1 is of interest, not only for gaining a better understanding of viral entry into a target cell, but also because it is directly linked to pathogenesis and clinical outcome.

The overall goal of this thesis was to study the role of CD4 binding for a primate lentivirus. To do this, we set out to develop an SIV that no longer required CD4 for entry, to assess the consequence of removing this interaction for the virus while simultaneously determining if there was any benefit for the infected host. Within the lentivirus family, primate lentiviruses have evolved most recently and CD4 tropism is unique to this group (6), suggesting a specific role for this interaction, either by increasing viral fitness and, or, making the virus-host exchange more favorable for the virus. The former could be achieved through an overall increase in avidity at the stage of virion binding to the cell through the engagement of two receptors rather than one. The latter is hypothesized to result from both the shielding of vulnerable epitopes on the viral Env from neutralizing antibodies, which are only exposed after CD4 engagement (7, 8), and the selective targeting of $CD4^+$ T cells, a critical component of the adaptive immune response (9–12).

Our work has shown that neither a CD4-independent virus nor a CD4-independent virus that no longer binds CD4 suffers from a severe fitness deficit, in vitro

or in vivo, suggesting that the gp120-CD4 binding interaction is not required for robust replication. The genetic mutations that arose to adapt the virus to growth in a CD4-negative cell line resulted in conformational changes within Env, as evidenced by an increase in neutralization sensitivity to antibodies targeting epitopes across gp120. However, in identifying the minimum set of mutations required to recapitulate the CD4-independent phenotype of our novel variant we discovered a strain, that while able to replicate in CD4-negative cells as efficiently as the parental strain, was not neutralization sensitive. These results indicate that the evolution to bind CD4 likely served as a mechanism by which the virus could "hide" from the host immune response, but that under at least one condition CD4-independence and neutralization sensitivity can be uncoupled. Finally, iMac239-ΔD385 infection in rhesus macaques did not deplete $CD4^+$ T cells, leaving this cell population intact to provide T cell help to the adaptive immune system, and resulting in a robust immune response capable of controlling viral replication.

Deriving CD4-independence in vitro

Chapter 2 details the derivation and characterization of a novel CD4-independent strain from the neutralization resistant, CD4-dependent, CCR5-tropic clone SIVmac239, termed iMac239. CD4-independence was achieved through selection in cell culture, which fixed mutations in the viral *env* gene. Selection for CD4-independence also resulted in a more "open" Env trimer conformation that was susceptible to antibodies targeting CD4-induced epitopes, even in the absence of CD4. Four mutations in gp120 were found to be sufficient to confer CD4 independence, with one mutation, D178G, in

the V1/V2 loop shown to be necessary for the phenotype. It is worth noting that many other cited examples of viruses capable of using low levels or no CD4 for entry, have identified various mutations in the V1/V2 region as drivers of the phenotype (13–19). Strikingly, the D178G mutation alone in the context of a replication competent SIVmac239 molecular clone imparted a severe fitness cost, suggesting that this mutation on its own either results in misfolding of the Env protein or causes a conformational change within the Env trimer that disrupts normal cellular entry. We have shown that a single additional C2 mutation, H224Q, just past the V1/V2 loops, is able to stabilize the D178G mutant virus, although these two mutations together do not result in a CD4-independent virus comparable to the parental iMac239; additional changes in V3 and V4 are required. Strikingly, the SIVmac239 variant carrying a minimum set of iMac239 changes to confer CD4-independence was not neutralization sensitive to a small panel of antibodies targeting CD4-induced epitopes, unlike other reported CD4-independent viruses. Since the mutations in this strain all occur in gp120, this result indicates that iMac239's neutralization sensitivity is due to the additional mutations in gp41. This finding is supported by the model of "intrinsic reactivity" published by Haim, et al.(20), in which mutations in gp41 can enhance gp120 neutralization sensitivity.

While this novel iMac239 strain is CD4-independent, none of the mutations in its Env are in the CD4-binding site, suggesting that this virus is still capable of binding CD4. Since we strove to study viral infection in the absence of CD4 binding we removed, through mutation, the Aspartic Acid residue at position 385 in the iMac239 Env. This residue is analogous to the Aspartic Acid at position 368 in the HIV-1 Env and has been shown to be an essential interaction between Env and CD4 (5, 21). This new strain,

iMac239-ΔD385, displayed no reduction in fitness in vitro compared to iMac239 in T cell lines or rhesus PBMCs. The same mutation introduced into SIVmac239 resulted in a virus that was unable to replicate. This result, along with the resistance of iMac239-ΔD385 to inhibition by soluble CD4, indicated that we had either completely ablated CD4 binding or reduced it significantly. Like iMac239, iMac239-ΔD385 is sensitive to neutralization, both by plasma samples from SIVmac251-infected animals and monoclonal antibodies to various Env epitopes.

As perhaps our strongest piece of evidence for true CD4-independent infection, we showed that iMac239 and iMac239-ΔD385 are capable of infecting $CD8^+$ T cells in stimulated rhesus PBMC cultures. The proportion of infected $CD8^+$ T cells in both infected cultures was too great for these cells to have been $CD4^+CD8^+$ T cells that downregulated CD4 upon infection. This result is clear proof of expansion of tropism to a $CD4^-CCR5^+$ cell type, which would only be possible with a true CD4-independent virus.

Future directions for in vitro characterization of iMac239 and iMac239-ΔD385

The importance of a mutation in the V1/V2 loop for other viruses capable of utilizing low levels of CD4 for entry (13–19) and our CD4-independent strain indicate that this region plays an important role in altering CD4 engagement. Since this region has not been implicated in the direct binding interaction (1, 22), it would seem that its influence occurs through a conformational change. Although there is not yet a crystal structure of the SIV Env trimer, either alone or engaged with rhesus CD4, further structural analysis is warranted to better understand the mechanism of trimer transition from a “closed”, CD4-unbound, state to an “open”, CD4-bound, state through genetic

mutation rather than CD4 binding. Cryo-electron tomography thus far has given us the best picture of the open and closed states of the SIV Env trimer (23, 24), utilization of this technique along with a panel of viruses including SIVmac239, iMac239, and various SIVmac239 strains carrying individual and combinations of iMac239 mutations should provide valuable information regarding the regional changes required for CD4-independence.

Our discovery that a molecular clone bearing solely the gp120 mutations from iMac239 recapitulated the CD4-independent phenotype, but not the neutralization sensitivity of the parental strain allows the two phenotypes to be uncoupled. This variant requires further testing in additional cell types, including rhesus PBMCs, to confirm that it does in fact replicate with the same kinetics and exhibits the same tropism as iMac239. If so, this virus could be an interesting strain to study in vivo as the first example of a neutralization resistant CD4-independent primate lentivirus. Additionally, the existence of this variant raises multiple questions, including why iMac239 developed the mutations in gp41 at all when they are not required for CD4-independence and how the mutations in gp41 alter the interaction between gp120 and gp41 to further change the conformation of the Env trimer and result in gp120 neutralization sensitivity.

In our study of iMac239-ΔD385 replication in stimulated rhesus PBMCs we observed a slight delay in the time of peak replication compared to SIVmac239 and iMac239, although the magnitude of the peak was similar for all three viruses. Of note, this delay in time of peak replication was not observed in our $CD4^+$ or $CD4^-$ T cell lines, where CCR5 expression is higher (unpublished) than that in rhesus PBMCs (25–28). These findings suggest differences in entry or replication kinetics that we have not

characterized. Time-of-addition experiments with a fusion inhibitor such as T-20, and studies characterizing the affinity of iMac and iMac239-ΔD385 for rhesus CCR5 could be used to determine how alterations in CD4-dependence affect fusion kinetics. Given Francella et al.'s (29) recent work showing CD4-independent Envs' reduced ability to use low levels of CCR5 and increased sensitivity to Maraviroc, it is likely our two CD4-independent variants have reduced CCR5 usage efficiency. While iMac239 may be able to overcome this defect by also using CD4 for entry, thereby increasing the overall avidity of the binding interaction between virion and cell, iMac239-ΔD385 cannot due to its ablated CD4 binding site, resulting in a delay in replication.

Finally, while we have shown clear evidence of $CD8^+$ T cell infection by both iMac239, and iMac239-ΔD385 in stimulated rhesus PBMCs, the novelty of this finding requires confirmation by other methods. One way to achieve this would be isolation of a pure $CD8^+$ T cell culture prior to infection with iMac239 and iMac239-ΔD385. Alternatively infected cells from whole rhesus PMBC cultures could be sorted at peak infection and then analyzed for mRNA expression of CD4 and CD8. Either method would prove infection of a $CD4^-$ $CD8^+$ T cell as opposed to a double positive ($CD4^+$ $CD8^+$) T cell that downregulated CD4 upon infection (30, 31).

Evaluation of iMac239-ΔD385 in vivo

We hypothesized that removing CD4 tropism from SIV in vivo would result in one of three outcomes. First, the virus would have a natural history similar to that of macrophage-tropic SIVs, which replicate to a low acute peak but then disseminate into tissues where they ultimately cause pathologies such as giant cell pneumonia and

encephalitis (32–36). Second, given a presumed expanded tropism to any cell expressing sufficient levels of CCR5 for entry, the virus would replicate to a very high acute peak and sustain high rates of viral replication throughout the chronic phase ultimately leading to early onset AIDS. Ortiz et al.'s (32) work showing that depletion of $CD4^+$ T cells prior to SIV infection resulted in an outgrowth of CD4-independent strains that were highly pathogenic supports this hypothesis. Third, we hypothesized that an intermediate outcome might be possible. In this case, the virus would replicate to a high acute peak but would ultimately be controlled down to low levels of replication during the chronic phase, similar to other live attenuated strains (37–41). Viral control would be the result of the adaptive immune system, which, now supported by $CD4^+$ T cells, was able to efficiently exploit viral neutralization sensitivity. As chapter 3 of this thesis details, the third outcome is what we observed, the virus replicated to an acute peak similar to that of wildtype but was then controlled to undetectable levels in the plasma, and extremely low levels in the tissues, likely as a result of a high and sustained humoral response effectively neutralizing this neutralization sensitive virus.

While our in vitro studies showed that iMac239-ΔD385 replicated to a similar peak as SIVmac239 in stimulated rhesus PBMCs, although with a slight delay, we did not know if the conformational changes required to make Env CD4-independent would impart a fitness cost in vivo. Thus, when we observed an acute plasma load viral peak that was similar to that of SIVmac239 in vivo it was important to perform SGA on the iMac239-ΔD385 replicating in animals to determine whether the mutations in the inoculum had changed in any way or if any additional mutations had arisen. We found that all of the original iMac239-ΔD385 mutations were intact at peak viremia and no

additional mutations were found in three of four animals. The fourth animal had a single additional amino acid change in Env in 40% of amplicons sequenced, however it is likely that this mutation was due to MHC-II mediated immune pressure, rather than an evolution to improve replicative fitness. These findings, along with previous studies (32–36), demonstrate that a CD4-independent strain is competent for in vivo replication.

We hypothesized that infection with a non-CD4 tropic virus would result in retargeting of the virus away from selective infection of $CD4^+$ T cells, although there was no reason these cells would not also be infected since they express CCR5. The flip side of this coin was that infecting animals with a CCR5-tropic virus meant that any cell type expressing sufficient levels of CCR5 would be susceptible to infection. We had already observed this possibility in vitro with the infection of $CD8^+$ T cells in stimulated RhPBMCs. Thus monitoring $CD4^+$ and $CD8^+$ T cell populations in vivo as well as the identity of infected cells in tissues was important to understand the tropism of the virus in a host. Unlike wildtype strains (4, 42, 43), iMac239-ΔD385-infected animals never experienced a decline in their $CD4^+$ T cell populations, either in the periphery or in the GALT. We also did not observe a decline in $CD8^+$ T cells in either compartment. Upon examination of tissues from peak infection we discovered $CD3^+$, $CD68^+$, and $CD3^-$, $CD2^-$, $CD68^-$, $CD163^-$, $LN5^-$, DR^-, $CD123^-$, $CD209^-$, $CD20^-$ infected cells in both the lamina propria and lymph node. Taken together, these results show that while T cells are certainly infected, significant portions of these cells are not being killed.

The undetectable levels of plasma viremia throughout the chronic phase of infection as well as the extremely low levels of viral RNA and DNA in the lymph nodes during chronic phase indicated the presence of host immune responses capable of

exquisite viral control. We evaluated both humoral and cellular responses in an attempt to determine if one or the other, or both could be the mechanism of control. We discovered that all four animals developed high and sustained neutralizing antibody responses to Tier-1 (neutralization sensitive) SIV strains as well as polyfunctional $CD4^+$ and $CD8^+$ T cell responses. Through a $CD8^+$ T cell depletion in two animals we surmised that the neutralizing antibody response was a major contributor to viral control given that the ensuing viral peak was low and transient during the time of the $CD8^+$ T cell depletion. While we were able to show that polyfunctional T cell responses were present in all four animals, we were not able to evaluate the potential of the T cell response to control viral replication in the absence of the neutralizing antibody response. The magnitude and maintenance of both of these immune responses is striking given the rarity of viral genetic material in the lymph node, which has been implicated as the main driver of T cell and B cell responses in other live attenuated SIV strains (37). These observations suggest that iMac239-ΔD385-infected animals were able to mount effective memory responses that were sustained even in the absence of, or with very low levels of, viral replication as the source of antigen.

Based on our observation of both polyfunctional T cell responses and high neutralizing antibody titers we tested whether iMac239-ΔD385 could serve as a live attenuated vaccine (LAV), which is one of two most successful SIV vaccination strategies tested to date (44–46). For the challenge study pathogenic SIVsmE660 swarm was inoculated intrarectally once a week for up to ten weeks in four iMac239-ΔD385 infected animals and four Trim5α matched naive controls. Given the small numbers of animals and the confounding factor of three separate Trim5α genotypes the results of this

study are anecdotal, although these preliminary findings suggest a trend towards improved challenge outcome in the vaccinated group.

While all four control animals were infected by the second challenge, regardless of Trim5α alleles, only two iMac239-ΔD385 animals were infected on the second challenge, one with the most permissive Trim5α genotype and one with the intermediate genotype. The two iMac239-ΔD385 animals carrying the most restrictive Trim5α genotype either did not become infected until the seventh challenge or remained uninfected after ten challenge inoculations. When compared by Trim5α genotype, iMac239-ΔD385 animals exhibited an approximate one log reduction in SIVsmE660 peak viral load. Additionally, two of three iMac239-ΔD385 maintained the majority of $CD4^+$ T cells in the lamina propria while control animals lost the majority of this cell population, similar to wildtype SIVsmE660 infection (47). As SIVsmE660 infection progressed in three iMac239-ΔD385 animals, there was clear selection for a predicted neutralization resistant SIVsmE660 strain, suggesting a humoral response in these animals that was exerting immune pressure on the virus. Indeed, evaluation of the neutralizing antibody response in these animals revealed that two of the three animals had developed a humoral response that had increased in magnitude and expanded in breadth as evidenced by the 100% neutralization of a Tier-2 SIVsmE660-2A5.IAKN strain, the first report of this occurrence (48).

Taken together, while prior iMac239-ΔD385 infection did not overtly prevent acquisition of a pathogenic heterologous challenge strain, the data suggest that the time to viral acquisition was slower when favorable Trim5α alleles were present, time to acute viral peak was slower, the magnitude of the viral peak was lower, and $CD4^+$ T cells were

preserved, indicating that with longer follow-up the overall outcome of iMac239-ΔD385 animals may be better. Finally, the increase in magnitude and expansion of breadth of the humoral response in two iMac239-ΔD385 animals post challenge raise the tantalizing possibility of the role of iMac239-ΔD385 as a prime for the adaptive immune response.

Future Directions for iMac239-ΔD385 in vivo studies

While this study was small, with only four rhesus macaques infected with iMac239-ΔD385, it has provided a wealth of information suggesting many avenues of exploration. One glaring unknown is the identity of the third infected cell type we observed at peak infection in tissue samples from the lymph node and lamina propria. Further investigation is required, either through testing larger panels of antibodies to other cellular markers using confocal microscopy, or through the sorting of infected cells from tissues and subsequent transcriptional analysis to determine the cellular identity based on mRNA expression patterns. One requirement of both of these methods is large quantities of tissues with sufficient numbers of infected cells to power studies for statistical significance. This requirement can only be met during the acute phase of iMac239-ΔD385 infection when there are high numbers of infected cells in tissues. Given the ethical guidelines on the number and size of tissue biopsies, sacrifice of a small number of animals at the peak infection time point would be the best method to acquire large amounts of tissues. Necropsy at this time would also allow for tissue sampling of other compartments such as the brain and lungs where more extensive replication in macrophages may occur.

Necropsying small numbers of animals at time points during the acute and early part of the chronic phases will not only provide more samples for the identification of the unknown infected cell type, but will also help in determining the primary locations of active viral replication during iMac239-ΔD385 infection. As of now, while we know that virus is present in the lymph node (albeit at very low levels) during the chronic phase of infection, we do not know if this is the sole site of replication, or if other compartments, such as the brain, may also harbor replicating virus. Therefore, sampling a variety of tissues from the time of peak viral replication through the point where plasma viral load is undetectable will show not only the sites of initial viral replication but also where virus resides in the face of a strong humoral response. For these experiments in situ hybridization for SIV RNA along with confocal microscopy to identify cells containing viral RNA would be useful for determining regions of viral replication within tissues. Isolation and sorting of cells from these regions with subsequent immunophenotyping and measurement of p27 Gag will further characterize the cell populations producing virus.

Long-term follow-up of iMac239-ΔD385-infected animals will be important to determine if the immune responses observed during chronic infection are sufficient to ultimately clear the virus. If so, this would be the second reported example of viral clearance, after Hansen et al.'s (46) data showing viral clearance as a result of RhCMV-vectored vaccination. It would be the first example of viral clearance resulting from immune responses developed during the course of active SIV infection and provide an avenue of investigation into another immune-mediated mechanism of clearance.

A major area of interest is the mechanism of control of iMac239-ΔD385 replication. Our current hypothesis is that host control is mediated by the humoral response, given the high neutralizing titers we observed, the extreme neutralization sensitivity of iMac239-ΔD385, and the low and transient peak observed after $CD8^+$ T cell depletion. However, this hypothesis does not exclude the possibility that other components of the adaptive immune system are capable of viral control. To ascertain which aspects of the immune system may be contributing to viral control, depletions of three key cell types must be considered: $CD4^+$ T cells, $CD8^+$ T cells, and B cells.

Depletion of $CD4^+$ T cells prior to iMac239-ΔD385 infection in a manner similar to that described by Ortiz et al. (32), would highlight the critical role of $CD4^+$ T cells in the development of the adaptive cellular and humoral responses. It is likely that in this scenario iMac239-ΔD385 will have a natural history similar to that observed by Ortiz et al. (32) with a high peak viral load during acute infection followed by high viral load set point and rapid progression to AIDS. This outcome would be due to the ability of iMac239-ΔD385 to replicate in non-$CD4^+$ cells and the inability of the adaptive immune to mount an effective response without $CD4^+$ T cell help.

Depletion of $CD8^+$ T cells during the course of iMac239-ΔD385 infection needs to be repeated to confirm our earlier findings in larger numbers of animals. Our preliminary evidence in two animals of a low and transient viral peak following depletion of peripheral $CD8^+$ T cells suggested that $CD8^+$ T cells are making only a minor contribution to viral control. Our current hypothesis is that when $CD8^+$ T cell immune pressure is relieved only a low amount of viral replication is possible because of the high levels of circulating neutralizing antibodies.

Depletion of B cells prior to and during the acute phase (49) of iMac239-ΔD385 infection would show whether the cellular arm of adaptive immunity is capable of viral control. The goal of this experiment is to prevent the host from developing its humoral response, which we believe is the primary source of viral control in this model. Our data showed that both $CD4^+$ and $CD8^+$ T cells had polyfunctional cytokine responses in both the periphery and lamina propria, however we do not yet know if these responses would be effective in controlling viral replication. If viral replication did not decrease significantly after the acute phase in this experiment, it would suggest that cellular responses alone are insufficient to mediate control. However, if the viral set point were significantly lower than the acute peak, more so than the decline seen in wild type infection, it would suggest that the cellular responses produced in iMac239-ΔD385 infection are sufficient to control viral replication and could work synergistically with the humoral response.

In all three of these depletion experiments any occurrence of moderate to high levels of viral replication would allow for the analysis of viral sequences to examine any viral evolution occurring in vivo. Any mutations that arise would serve as genetic signatures and provide information on whether the virus is adapting to improve fitness or escape immune pressure. It would also be interesting to determine if iMac239-ΔD385 reacquired the ability to bind CD4, either by reinserting an aspartic acid residue at position 385 or by developing compensatory mutations. If CD4 tropism is reacquired, this virus could be tested in naïve animals to determine if the reacquisition served to improve viral fitness and/or shield the virus from a particular arm of the immune response, as

evidenced by higher rates of acute viral replication in acute infection, or the inability of the host to control viral replication in the chronic phase, respectively.

Another important experiment would be to infect naïve rhesus macaques with the parental CD4-independent iMac239 strain, which still has an intact CD4 binding site. We have already established that iMac239 is capable of CD4-independent infection in vitro with the observation that it infects stimulated rhesus macaque $CD8^+$ T cells, thus we have reason to believe that it too would exhibit an expanded tropism in vivo. Also like iMac239-ΔD385, iMac239 has an open Env trimer conformation and is globally sensitive to monoclonal antibodies targeting epitopes across gp120; therefore we hypothesize that this virus would also be neutralized by the host humoral response. The key, and only, difference between the two viruses is the binding interaction between gp120 and the CD4 molecule. Thus any alterations in the natural history of iMac239 infection compared to that of iMac239-ΔD385 would be a consequence of Env-CD4 binding.

Previous work has shown that binding of gp120 to CD4 suppresses T cell activation and proliferation in response to antigen (50–52) . Additionally, the binding of the viral and cellular proteins to one another impedes the ability of CD4 to bind to MHC class II, thereby disrupting the natural function of CD4 (1, 53, 54). The extent to which the binding interaction disrupts $CD4^+$ T cell function in vivo remains unknown. Comparison of $CD4^+$ T cell population dynamics, including proliferation, activation, and functionality, between iMac239-ΔD385 and iMac239 infections will illuminate whether the binding of gp120 to CD4 significantly affects the $CD4^+$ T cell population, and subsets, as a whole.

Further challenge studies should be performed to extend our preliminary findings in this small set of animals. Historically, live attenuated vaccines (LAVs) are much better at protecting against homologous challenge than heterologous challenge (45, 47, 55, 56) and it remains to be seen whether iMac239-ΔD385 has the ability to induce immune responses capable of protecting against a homologous SIVmac239 or SIVmac251 challenge. If further challenge studies are conducted using the SIVsmE660 stock, it will be critical to account for Trim5α alleles in the study population and group animals accordingly, either by choosing to use macaques that all have the same Trim5α alleles or by including sufficient numbers of control and iMac239-ΔD385 animals with each set of Trim5α alleles, so that Trim5α is not a confounding factor as it was in this initial study.

The recent creation of recombinant soluble Env trimers that adopt a native conformation through the SOSIP construct (57–59) has allowed for development of Env protein vaccination regimens that expose the immune system to the Env trimer in its natural, unliganded state, a previously unmet challenge (60). Current work on SOSIP trimers has focused on developing genetically diverse panels of trimers as well as determining the proper Env conformation to induce Tier-2 neutralizing antibodies post vaccination. The former has proven an easier hurdle to overcome with reports in the last year detailing SOSIP Env trimers made from a variety of HIV-1 clades (61, 62). Attempts to induce Tier-2 neutralizing antibodies have met with only moderate success (63). Based on our finding that iMac239-ΔD385 infection may have served as a prime for the host humoral response to develop robust Tier-2 neutralizing antibodies after exposure to a CD4-dependent pathogenic challenge, inclusion of a CD4-indendent SOSIP trimer in a sequential vaccination regimen may be the key to induce a broad neutralizing response.

The goal of this thesis was to gain a better understanding of why CD4 usage is an invariant feature of primate lentiviruses. Through the development of a true CD4-independent SIV, we have found that such a virus is capable of robust acute in vivo replication while sparing $CD4^+$ T cells. However, the virus ultimately succumbs to host control, likely as the result of increased Env neutralization sensitivity, a consequence of CD4-independent trimer conformations. Thus, our work indicates that the primary roles for CD4 engagement in primate lentiviral infection are 1) the ability to adopt conformations to shield vulnerable epitopes and 2) to drive pathogenesis by depleting $CD4^+$ T cells and disabling the adaptive immune system.

References

1. **Kwong PD**, **Wyatt R**, **Robinson J**, **Sweet RW**, **Sodroski J**, **Hendrickson WA**. 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393**:648–59.

2. **Wyatt R**, **Kwong PD**, **Desjardins E**, **Sweet RW**, **Robinson J**, **Hendrickson WA**, **Sodroski JG**. 1998. The Antigenic Structure of the HIV gp120 Envelope Glycoprotein. Nature **393**:705–711.

3. **Wilen CB**, **Tilton JC**, **Doms RW**. 2012. HIV: Cell Binding and Entry, p. 23–36. *In* HIV From Biology to Prevention and Treatment.

4. **Lackner AA**, **Lederman MM**, **Rodriguez B**. 2012. HIV pathogenesis: the host, p. 193–216. *In* HIV From Biology to Prevention and Treatment.

5. **Olshevsky U**, **Helseth E**, **Furman C**, **Li J**, **Haseltine W**, **Sodroski J**. 1990. Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. J Virol **64**:5701–7.

6. **Gifford RJ**. 2012. Viral evolution in deep time: Lentiviruses and mammals. Trends Genet **28**:89–100.

7. **Labrijn AF**, **Poignard P**, **Raja A**, **Zwick MB**, **Delgado K**, **Franti M**, **Binley J**, **Vivona V**, **Grundner C**, **Huang C-C**, **Venturi M**, **Petropoulos CJ**, **Wrin T**, **Dimitrov DS**, **Robinson J**, **Kwong PD**, **Wyatt RT**, **Sodroski J**, **Burton DR**. 2003. Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. J Virol **77**:10557–10565.

8. **Decker JM**, **Bibollet-Ruche F**, **Wei X**, **Wang S**, **Levy DN**, **Wang W**, **Delaporte E**, **Peeters M**, **Derdeyn CA**, **Allen S**, **Hunter E**, **Saag MS**, **Hoxie JA**, **Hahn BH**, **Kwong PD**, **Robinson JE**, **Shaw GM**. 2005. Antigenic conservation and immunogenicity of the HIV coreceptor binding site. J Exp Med **201**:1407–1419.

9. **Kalams SA**, **Walker BD**. 1998. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. J Exp Med **188**:2199–2204.

10. **Onabajo OO**, **Mattapallil JJ**. 2013. Expansion or Depletion of T Follicular

Helper cells During HIV Infection: Consequences for B cell Responses. Curr HIV Res **11**:595–600.

11. **Heeney JL**. 2002. The critical role of CD4+ T-cell help in immunity to HIV. Vaccine **20**:1961–1963.

12. **Altfeld M**, **Rosenberg ES**. 2000. The role of CD4(+) T helper cells in the cytotoxic T lymphocyte response to HIV-1. Curr Opin Immunol **12**:375–80.

13. **LaBranche CC**, **Hoffman TL**, **Romano J**, **Haggarty BS**, **Edwards TG**, **Matthews TJ**, **Doms RW**, **Hoxie JA**. 1999. Determinants of CD4 independence for a human immunodeficiency virus type 1 variant map outside regions required for coreceptor specificity. J Virol **73**:10310–9.

14. **Puffer BA**, **Altamura LA**, **Pierson TC**, **Doms RW**. 2004. Determinants within gp120 and gp41 contribute to CD4 independence of SIV Envs. Virology **327**:16–25.

15. **Kolchinsky P**, **Kiprilov E**, **Bartley P**, **Rubinstein R**, **Sodroski J**. 2001. Loss of a Single N-Linked Glycan Allows CD4-Independent Human Immunodeficiency Virus Type 1 Infection by Altering the Position of the gp120 V1 / V2 Variable Loops. J Virol **75**:3435–3443.

16. **Musich T**, **Peters PJ**, **Duenas-Decamp MJ**, **Gonzalez-Perez MP**, **Robinson J**, **Zolla-Pazner S**, **Ball JK**, **Luzuriaga K**, **Clapham PR**. 2011. A Conserved Determinant in the V1 Loop of HIV-1 Modulates the V3 Loop To Prime Low CD4 Use and Macrophage Infection. J Virol **85**:2397–405.

17. **Luciw PA**, **Shaw KE**, **Unger RE**, **Planelles V**, **Stout MW**, **Lackner JE**, **Pratt-Lowe E**, **Leung NJ**, **Banapour B**, **Marthas ML**. 1992. Genetic and biological comparisons of pathogenic and nonpathogenic molecular clones of simian immunodeficiency virus (SIVmac). AIDS Res Hum Retroviruses **8**:395–402.

18. **Reeves JD**, **Schulz TF**. 1997. The CD4-independent tropism of human immunodeficiency virus type 2 involves several regions of the envelope protein and correlates with a reduced activation threshold for envelope-mediated fusion. J Virol **71**:1453–1465.

19. **Yen P-J**, **Herschhorn A**, **Haim H**, **Salas I**, **Gu C**, **Sodroski J**, **Gabuzda D**. 2014. Loss of a conserved N-linked glycosylation site in the simian immunodeficiency

virus envelope glycoprotein V2 region enhances macrophage tropism by increasing CD4-independent cell-to-cell transmission. J Virol **88**:5014–28.

20. **Haim H**, **Strack B**, **Kassa A**, **Madani N**, **Wang L**, **Courter JR**, **Princiotto A**, **McGee K**, **Pacheco B**, **Seaman MS**, **Smith AB**, **Sodroski J**. 2011. Contribution of intrinsic reactivity of the HIV-1 envelope glycoproteins to CD4-independent infection and global inhibitor sensitivity. PLoS Pathog **7**(6): e1002101. doi:10.1371/journal.ppat.1002101.

21. **Li Y**, **Migueles SA**, **Welcher B**, **Svehla K**, **Phogat A**, **Louder MK**, **Wu X**, **Shaw GM**, **Connors M**, **Wyatt RT**, **Mascola JR**. 2007. Broad HIV-1 neutralization mediated by CD4-binding site antibodies. Nat Med **13**:1032–1034.

22. **Finzi A**, **Pacheco B**, **Xiang S-H**, **Pancera M**, **Herschhorn A**, **Wang L**, **Zeng X**, **Desormeaux A**, **Kwong PD**, **Sodroski J**. 2012. Lineage-Specific Differences between Human and Simian Immunodeficiency Virus Regulation of gp120 Trimer Association and CD4 Binding. J Virol **86**:8974–8986.

23. **White TA**, **Bartesaghi A**, **Borgnia MJ**, **de la Cruz MJV**, **Nandwani R**, **Hoxie JA**, **Bess JW**, **Lifson JD**, **Milne JLS**, **Subramaniam S**. 2011. Three-Dimensional Structures of Soluble CD4-Bound States of Trimeric Simian Immunodeficiency Virus Envelope Glycoproteins Determined by Using Cryo-Electron Tomography. J Virol **85**:12114–12123.

24. **White TA**, **Bartesaghi A**, **Borgnia MJ**, **Meyerson JR**, **de la Cruz MJV**, **Bess JW**, **Nandwani R**, **Hoxie JA**, **Lifson JD**, **Milne JLS**. 2010. Molecular Architectures of Trimeric SIV and HIV-1 Envelope Glycoproteins on Intact Viruses : Strain- Dependent Variation in Quaternary Structure. PLoS Pathog **6**(12): e1001249. doi:10.1371/journal.ppat.1001249.

25. **Joseph SB**, **Arrildt KT**, **Swanstrom AE**, **Schnell G**, **Lee B**, **Hoxie JA**, **Swanstrom R**. 2014. Quantification of Entry Phenotypes of Macrophage-Tropic HIV-1 across a Wide Range of CD4 Densities. J Virol **88**:1858–1869.

26. **Lee B**, **Sharron M**, **Montaner LJ**, **Weissman D**, **Doms RW**. 1999. Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. Proc Natl Acad Sci U S A **96**:5215–20.

27. **Loftin LM**, **Kienzle MF**, **Yi Y**, **Lee B**, **Lee F**, **Gorry PR**, **Collman RG**. 2011. Constrained use of CCR5 on CD4+ lymphocytes by R5X4 HIV-1: Efficiency of

Env-CCR5 interactions and low CCR5 expression determine a range of restriced CCR5-mediated entry. Virology **402**:135–148.

28. **Mummidi S**, **Adams LM**, **VanCompernolle SE**, **Kalkonde M**, **Camargo JF**, **Kulkarni H**, **Bellinger AS**, **Bonello G**, **Tagoh H**, **Ahuja SS**, **Unutmaz D**, **Ahuja SK**. 2007. Production of specific mRNA transcripts, usage of an alternate promoter, and octamer-binding transcription factors influence the surface expression levels of the HIV coreceptor CCR5 on primary T cells. J Immunol **178**:5668–5681.

29. **Francella N**, **Elliott ST**, **Yi Y**, **Gwyn SE**, **Ortiz AM**, **Li B**, **Silvestri G**, **Paiardini M**, **Derdeyn C a**, **Collman RG**. 2013. Decreased plasticity of coreceptor use by CD4-independent SIV Envs that emerge in vivo. Retrovirology **10**:133.

30. **Piguet V**, **Schwartz O**, **Le Gall S**, **Trono D**. 1999. The downregulation of CD4 and MHC-I by primate lentiviruses: a paradigm for the modulation of cell surface receptors. Immunol Rev **168**:51–63.

31. **Kirchhoff F**, **Schindler M**, **Specht A**, **Arhel N**, **Münch J**. 2008. Role of Nef in primate lentiviral immunopathogenesis. Cell Mol Life Sci **65**:2621–2636.

32. **Ortiz AM**, **Klatt NR**, **Li B**, **Yi Y**, **Tabb B**, **Hao XP**, **Sternberg L**, **Lawson B**, **Carnathan PM**, **Cramer EM**, **Engram JC**, **Little DM**, **Ryzhova E**, **Gonzalez-scarano F**, **Paiardini M**, **Ansari AA**, **Ratcliffe S**, **Else JG**, **Brenchley JM**, **Collman RG**, **Estes JD**, **Derdeyn CA**, **Silvestri G**. 2011. Depletion of CD4 + T cells abrogates post-peak decline of viremia in SIV-infected rhesus macaques. J Clin Invest **121**:4433–45.

33. **Micci L**, **Alvarez X**, **Iriele RI**, **Ortiz AM**, **Ryan ES**, **McGary CS**, **Deleage C**, **McAtee BB**, **He T**, **Apetrei C**, **Easley K**, **Pahwa S**, **Collman RG**, **Derdeyn CA**, **Davenport MP**, **Estes JD**, **Silvestri G**, **Lackner AA**, **Paiardini M**. 2014. CD4 Depletion in SIV-Infected Macaques Results in Macrophage and Microglia Infection with Rapid Turnover of Infected Cells. PLoS Pathog **10**(10): e1004467. doi:10.1371/journal.ppat.1004467.

34. **Flaherty MT**, **Hauer DA**, **Mankowski JL**, **Zink MC**, **Clements JE**. 1997. Molecular and biological characterization of a neurovirulent molecular clone of simian immunodeficiency virus. J Virol **71**:5790–8.

35. **Mankowski JL**, **Flaherty MT**, **Spelman JP**, **Hauer DA**, **Didier PJ**, **Amedee AM**, **Murphey-Corb M**, **Kirstein LM**, **Muñoz A**, **Clements JE**, **Zink MC**.

1997. Pathogenesis of simian immunodeficiency virus encephalitis: viral determinants of neurovirulence. J Virol **71**:6055–60.

36. **Marthas M**, **Banapour B**, **Sutjipto S**, **Siegel M**, **Marx P**, **Gardner M**, **Pedersen N**, **Luciw P**. 1989. Rhesus macaques inoculated with molecularly cloned simian immunodeficiency virus. J Med Primatol **18**:311–9.

37. **Fukazawa Y**, **Park H**, **Cameron MJ**, **Lefebvre F**, **Lum R**, **Coombes N**, **Mahyari E**, **Hagen S**, **Bae JY**, **Reyes III MD**, **Swanson T**, **Legasse AW**, **Sylwester A**, **Hansen SG**, **Smith AT**, **Stafova P**, **Shoemaker R**, **Li Y**, **Oswald K**, **Axthelm MK**, **McDermott A**, **Ferrari G**, **Montefiori DC**, **Edlefsen PT**, **Piatak Jr. M**, **Lifson JD**, **Sekaly RP**, **Picker LJ**. 2012. Lymph node T cell responses predict the efficacy of live attenuated SIV vaccines. Nat Med **18**:1673–1681.

38. **Kestler HW**, **Ringler DJ**, **Mori K**, **Panicali DL**, **Sehgal PK**, **Daniel MD**, **Desrosiers RC**. 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell **65**:651–662.

39. **Johnson RP**, **Lifson JD**, **Czajak SC**, **Cole KS**, **Manson KH**, **Glickman R**, **Yang J**, **Montefiori DC**, **Montelaro R**, **Wyand MS**, **Desrosiers RC**. 1999. Highly attenuated vaccine strains of simian immunodeficiency virus protect against vaginal challenge: inverse relationship of degree of protection with level of attenuation. J Virol **73**:4952–4961.

40. **Lackner AA**, **Vogel P**, **Ramos RA**, **Kluge JD**, **Marthas M**. 1994. Early events in tissues during infection with pathogenic (SIVmac239) and nonpathogenic (SIVmac1A11) molecular clones of simian immunodeficiency virus. Am J Pathol **145**:428–439.

41. **Chakrabarti L**, **Baptiste V**, **Khatissian E**, **Cumont MC**, **Aubertin AM**, **Montagnier L**, **Hurtrel B**. 1995. Limited viral spread and rapid immune response in lymph nodes of macaques inoculated with attenuated simian immunodeficiency virus. Virology **213**:535–548.

42. **Veazey RS**, **DeMaria M**, **Chalifoux L V**, **Shvetz DE**, **Pauley DR**, **Knight HL**, **Rosenzweig M**, **Johnson RP**, **Desrosiers RC**, **Lackner AA**. 1998. Gastrointestinal Tract as a Major Site of CD4+ T Cell Depletion and Viral Replication in SIV Infection. Science **280**:427–431.

43. **Veazey RS**, **Mansfield KG**, **Tham IC**, **Carville AC**, **Shvetz DE**, **Forand AE**,

Lackner AA. 2000. Dynamics of CCR5 expression by CD4(+) T cells in lymphoid tissues during simian immunodeficiency virus infection. J Virol **74**:11001–7.

44. **Lifson JD**, **Haigwood NL**. 2012. Lessons in nonhuman primate models for AIDS vaccine research: from minefields to milestones., p. 463–482. *In* HIV From Biology to Prevention and Treatment.

45. **Koff WC**, **Johnson PR**, **Watkins DI**, **Burton DR**, **Lifson JD**, **Hasenkrug KJ**, **McDermott AB**, **Schultz A**, **Zamb TJ**, **Boyle R**, **Desrosiers RC**. 2006. HIV vaccine design: insights from live attenuated SIV vaccines. Nat Immunol **7**:19–23.

46. **Hansen SG**, **Jr MP**, **Ventura AB**, **Hughes CM**, **Gilbride M**, **Ford JC**, **Oswald K**, **Shoemaker R**, **Li Y**, **Matthew S**, **Gilliam AN**, **Xu G**, **Whizin N**, **Burwitz BJ**, **Planer L**, **Turner JM**, **Legasse AW**, **Axthelm MK**, **Nelson JA**, **Früh K**, **Sacha JB**, **Estes JD**, **Keele BF**, **Edlefsen PT**, **Lifson JD**, **Picker LJ**. 2014. Immune clearance of highly pathogenic SIV infection. Nature **502**: doi:10.1038/nature12519.

47. **Wyand MS**, **Manson K**, **Montefiori DC**, **Lifson JD**, **Johnson RP**, **Desrosiers RC**. 1999. Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. J Virol **73**:8356–8363.

48. **Roederer M**, **Keele BF**, **Schmidt SD**, **Mason RD**, **Welles HC**, **Fischer W**, **Labranche C**, **Foulds KE**, **Louder MK**, **Yang Z-Y**, **Todd J-PM**, **Buzby AP**, **Mach L V.**, **Shen L**, **Seaton KE**, **Ward BM**, **Bailer RT**, **Gottardo R**, **Gu W**, **Ferrari G**, **Alam SM**, **Denny TN**, **Montefiori DC**, **Tomaras GD**, **Korber BT**, **Nason MC**, **Seder RA**, **Koup RA**, **Letvin NL**, **Rao SS**, **Nabel GJ**, **Mascola JR**. 2014. Immunological and Virological Mechanisms of Vaccine-Mediated Protection Against SIV and HIV. Nature **505**:502–508.

49. **Miller CJ**, **Genescà M**, **Abel K**, **Montefiori D**, **Forthal D**, **Bost K**, **Li J**, **Favre D**, **McCune JM**. 2007. Antiviral antibodies are necessary for control of simian immunodeficiency virus replication. J Virol **81**:5024–35.

50. **Chirmule N**, **Kalyanaraman VS**, **Oyaizu N**, **Slade HB**, **Pahwa S**. 1990. Inhibition of functional properties of tetanus antigen-specific T-cell clones by envelope glycoprotein GP120 of human immunodeficiency virus. Blood **75**:152–159.

51. **Cefai D**, **Debre P**, **Kaczorek M**, **Autran B**, **Bismuth G**. 1990. Human

Immunodeficiency Virus-1 glycoproteins gpl2O and gp160 specifically inhibit the CD3 / T cell-antigen receptor phosphoinositide transduction pathway. J Clin Invest **86**:2117–2124.

52. **Schwartz O**, **Alizon M**, **Heard J-M**, **Danos L**. 1994. Impairment of T cell receptor-dependent stimulation in CD4+ lymphocytes after contact with membrane-bound HIV-1 envelope glycoprotein. Virology **198**:360–365.

53. **Arthos J**, **Deen KC**, **Chaikin IM**, **Fornwald JA**, **Sathe G**, **Sattentau QJ**, **Clapham PR**, **Weiss RA**, **McDougal JS**, **Pietropaolo C**. 1989. Identification of the residues in human CD4 critical for the binding of HIV. Cell **57**:469–481.

54. **Clayton LK**, **Sieh M**, **Pious DA**, **Reinherz EL**. 1989. Identification of human CD4 residues affecting class II MHC versus HIV-1 gp120 binding. Nature **339**:548–51.

55. **Whitney JB**, **Ruprecht RM**. 2004. Live attenuated HIV vaccines: pitfalls and prospects. Curr Opin Infect Dis **17**:17–26.

56. **Johnson RP**, **Desrosiers RC**. 1998. Protective immunity induced by live attenuated simian immunodeficiency virus. Curr Opin Immunol **10**:436–43.

57. **Lyumkis D**, **Julien J-P**, **de Val N**, **Cupo A**, **Potter CS**, **Klasse PJ**, **Burton DR**, **Sanders RW**, **Moore JP**, **Carragher B**, **Wilson IA**, **Ward AB**. 2013. Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 Env trimer. Science **342**:1484–1490.

58. **Julien J-P**, **Cupo A**, **Sok D**, **Stanfield RL**, **Lyumkis D**, **Deller MC**, **Klasse P-J**, **Burton DR**, **Sanders RW**, **Moore JP**, **Ward AB**, **Wilson IA**. 2013. Crystal structure of a soluble cleaved HIV-1 envelope trimer. Science **342**: doi:10.1126/science.1245625.

59. **Sanders RW**, **Derking R**, **Cupo A**, **Julien J-P**, **Yasmeen A**, **de Val N**, **Kim HJ**, **Blattner C**, **de la Peña AT**, **Korzun J**, **Golabek M**, **de Los Reyes K**, **Ketas TJ**, **van Gils MJ**, **King CR**, **Wilson IA**, **Ward AB**, **Klasse PJ**, **Moore JP**. 2013. A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. PLoS Pathog **9**(9): e1003618. doi:10.1371/journal.ppat.1003618.

60. **Sanders RW**, **van Gils MJ**, **Derking R**, **Sok D**, **Ketas TJ**, **Burger JA**,

Ozorowski G, **Cupo A**, **Simonich C**, **Goo L**, **Arendt H**, **Kim HJ**, **Lee JH**, **Pugach P**, **Williams M**, **Debnath G**, **Moldt B**, **van Breemen MJ**, **Isik G**, **Medina-Ramirez M**, **Back JW**, **Koff WC**, **Julien J-P**, **Rakasz EG**, **Seaman MS**, **Guttman M**, **Lee KK**, **Klasse PJ**, **LaBranche C**, **Schief WR**, **Wilson IA**, **Overbaugh J**, **Burton DR**, **Ward AB**, **Montefiori DC**, **Dean H**, **Moore JP**. 2015. HIV-1 neutralizing antibodies induced by native-like envelope trimers. Science **349**: doi:10.1126/science.aac4223.

61. **Pugach P**, **Ozorowski G**, **Cupo A**, **Ringe R**, **Yasmeen A**, **de Val N**, **Derking R**, **Kim HJ**, **Korzun J**, **Golabek M**, **de los Reyes K**, **Ketas TJ**, **Julien J-P**, **Burton DR**, **Wilson IA**, **Sanders RW**, **Klasse PJ**, **Ward AB**, **Moore JP**. 2015. A Native-Like SOSIP.664 Trimer Based on an HIV-1 Subtype B *env* Gene. J Virol **89**:3380–3395.

62. **Julien J**, **Hyun J**, **Ozorowski G**, **Hua Y**, **Torrents de la Pena A**, **DeTaeye S**, **Nieusma T**, **Cupo A**, **Yasmeen A**, **Golabek M**, **Pugach P**, **Klasse PJ**, **Moore JP**, **Sanders RW**, **Ward AB**, **Wilson IA**. 2015. Design and structure of two HIV-1 clade C SOSIP . 664 trimers that increase the arsenal of native-like Env immunogens. PNAS **112**:11947–11952.

63. **Georgiev IS**, **Joyce MG**, **Yang Y**, **Sastry M**, **Zhang B**, **Baxa U**, **Chen RE**, **Druz A**, **Lees CR**, **Narpala S**, **Schön A**, **Van Galen J**, **Chuang G-Y**, **Gorman J**, **Harned A**, **Pancera M**, **Stewart-Jones GBE**, **Cheng C**, **Freire E**, **McDermott AB**, **Mascola JR**, **Kwong PD**. 2015. Single-chain soluble BG505.SOSIP gp140 trimers as structural and antigenic mimics of mature closed HIV-1 Env. J Virol **89**:5318-5329.